Direct Involvement of Yeast Type I Myosins in Cdc42-dependent Actin Polymerization

Terry Lechler,* Anna Shevchenko,‡ Andrej Shevchenko,‡ and Rong Li*  

*Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115; and ‡ Peptide and Protein Group, European Molecular Biology Laboratory (EMBL), 69012 Heidelberg, Germany

Abstract. The generation of cortical actin filaments is necessary for processes such as cell motility and cell polarization. Several recent studies have demonstrated that Wiskott-Aldrich syndrome protein (WASP) family proteins and the actin-related protein (Arp) 2/3 complex are key factors in the nucleation of actin filaments in diverse eukaryotic organisms. To identify other factors involved in this process, we have isolated proteins that bind to Bee1p/Las17p, the yeast WASP-like protein, by affinity chromatography and mass spectroscopic analysis. The yeast type I myosins, M yo3p and M yo5p, have both been identified as Bee1p-interacting proteins. Like Bee1p, these myosins are essential for cortical actin assembly as assayed by in vitro reconstitution of actin nucleation sites in permeabilized yeast cells. Analysis using this assay further demonstrated that the motor activity of these myosins is required for the polymerization step, and that actin polymerization depends on phosphorylation of myosin motor domain by p21-activated kinases (PAKs), downstream effectors of the small guanosine triphosphatase, Cdc42p. The type I myosins also interact with the Arp2/3 complex through a sequence at the end of the tail domain homologous to the Arp2/3-activating region of WASP-like proteins. Combined deletions of the Arp2/3-interacting domains of Bee1p and the type I myosins abolish actin nucleation sites at the cortex, suggesting that these proteins function redundantly in the activation of the Arp2/3 complex.

Key words: myosin • actin • Cdc42 • actin-related protein (Arp) 2/3 complex • Wiskott-Aldrich syndrome protein (WASP)

Introduction

Type I myosins are highly conserved proteins that appear to play roles in numerous processes, such as endocytosis, membrane trafficking, contractility, and cell motility (Mooseker and Cheney, 1995). In some eukaryotic organisms, these proteins localize to the leading edge of motile cells and are speculated to power membrane protrusion (Fukui et al., 1989; Wagner et al., 1992). The motor activity of type I myosins in amoebae depends on phosphorylation of the motor domain by p21-activated kinases (PAKs), downstream targets of Rho family GTPases (Maruta and Korn, 1977; Brzeska et al., 1997, 1999). The same phosphorylation site, known as the TEDS-rule phosphorylation site, also exists in yeast type I myosins and type VI myosins of mammalian cells (Wu et al., 1997; Buss et al., 1998), but the in vivo significance of this phosphorylation in Rho family GTPase-regulated cellular processes has not been demonstrated.

Budding yeast contains two type I myosins, M yo3p and M yo5p, which are functionally redundant in vivo (Goodson et al., 1996). These myosins consist of an NH2-terminal motor domain with F-actin binding and ATPase activity and IQ motifs for binding to a light chain. The COOH-terminal tail of the type I myosins includes a putative lipid-binding domain, a second F-actin binding domain (TH2 domain), an Src homology domain 3 (SH3) domain, and an acidic tail motif (see Results). Therefore, the tail has the potential to interact with a variety of proteins and membrane lipids. Double knockout of M Y O 3 and M Y O 5 results in defects in polarized cell growth and endocytosis, but it is not known how the myosins participate in these actin-based processes. In vitro, these myosins are phosphorylated at the conserved site by Ste20p and Cla4p, two yeast PAKs (Wu et al., 1997). Phosphorylation is required for function in vivo, as a nonphosphorylatable M yo3p mu-
Cdc42 is required for cell polarity and actin polymerization in a number of systems. A requirement for Cdc42 activity in actin assembly has been shown in permeabilized yeast cells, and A. canthamoeba, Dictyostelium, X. enopus, and neutrophil extracts (Li et al., 1995; Zigmond et al., 1997; Ma et al., 1998; Mullins and Pollard, 1999). The mechanisms by which Cdc42 promotes actin assembly were the subject of recent research. In both Dictyostelium and X. enopus egg extracts, the actin-related protein (Arp) 2/3 complex is required for Cdc42-induced actin assembly (Ma et al., 1999; Mullins and Pollard, 1999). This highly conserved seven-polypeptide complex is thought to catalyze the de novo nucleation of actin filaments, the rate-limiting step in actin polymerization (Cooper et al., 1983; Tobacman and Korn, 1983). It is also required for the actin polymerization-driven motility of pathogenic bacteria such as Listeria monocytogenes and Shigella flexneri (Welch et al., 1997; Gile et al., 1999). The nucleation activity of purified Arp2/3 complex is low but can be greatly stimulated by the Listeria actA protein (Welch et al., 1998), and by members of the Wiskott-Aldrich syndrome protein (WASP) family, including WA SP, neuronal (N)-WA SP, WA VE SCAR1, and Bee1p/lastp (Machesky et al., 1999; Rohatgi et al., 1999; Winter et al., 1999a; Yarar et al., 1999). This activation is dependent upon a COOH-terminal acidic domain conserved among all WA SP family members (Machesky and Insall, 1998). Both WA SP and N-WA SP contain a Cdc42-binding motif (A spenstrom et al., 1996; Miki et al., 1996; Symons et al., 1996). It has been shown that the ability of N-WA SP to activate the nucleation activity of the Arp2/3 complex is dependent upon binding to Cdc42p (Rohatgi et al., 1999). It is believed that Cdc42 binding induces a conformational change in N-WA SP, exposing its Arp2/3 activation domain. WA VE and Bee1p lack the Cdc42-interacting domain, and how they link signal transduction pathways to actin assembly is not yet known.

Our study started from an attempt to define functional components of cortical actin assembly sites using an in vitro reconstitution assay (Lechler and Li, 1997). In this assay, a Cdc42-dependent actin nucleation activity associated with cortical patches in permeabilized yeast cells was first eliminated by treatment with 2 M urea and reconstituted by incubation with a cytoplasmic extract. The extract was then washed away and actin polymerization at cortical patches was assayed after the addition of rhodamine-labeled actin monomers (G-actin). This approach led to the identification of Bee1p, a yeast member of the WA SP family, as one of the proteins required for the first step of the reconstitution (Lechler and Li, 1997). However, the lack of a Cdc42-binding motif suggested that signaling to actin polymerization was occurring in a different manner in this system. Furthermore, deletion of the acidic Arp2/3-binding domain of Bee1p has little effect on actin assembly and cell growth, suggesting that Bee1p is unlikely to be the sole activator of the Arp2/3 complex (Winter et al., 1999a). In this study, we first identified the yeast type I myosins as Bee1p-interacting proteins that are also required for cortical actin assembly in the permeabilized cells. Subsequent analysis provided evidence that these myosins have multiple roles in cortical actin assembly: they mediate Cdc42 regulation of actin polymerization through motor domain phosphorylation; and they function redundantly with Bee1p in the activation of the Arp2/3 complex.

Materials and Methods

Plasmid Construction

The myo5ΔC-hemagglutinin (HA) construct was prepared by PCR of the promoter (~284) and the first 1169 codons of MYO5 using genomic DNA and the primers 5’-GGCGGGCGCGCGGCGGGTGGTTTTAATCTCGTCGG-3’ and 5’-GGCGCGGGGAGTTCTGTTAACAAGTTCGACGG-3’. The product was digested with NolI and BamHI and ligated into PRL129, an HA-tagging vector derived from PRS306. The Myo5A-HA construct was generated by PCR using the primers 5’-GGCGGGGCGCGCGGGCCTGGGGAAAGAAGCTTACCG-3’ and 5’-GGCGGCAGAATTCGTTGGGAGCTTACGGACGGC-3’. The product was digested with NolI and EcoRI and ligated into PRL129. The glutathione S-transferase (GST)-Myo3A-expressing plasmid was made by PCR of the acidic domain of MYO3 using the primers 5’-GGCGGCGGAAATTCGTTGGGAGCTTACGGACGGC-3’ and 5’-GGCGGGCGGGAATTCACTCTGTATAATCAGTTACGGACGGC-3’. The product was cloned into the EcoRI site of pGEX-4T-1. The GST-Myo5A construct was created by subcloning of the Myo5p acidic domain. Full-length MYO5 was subjected to PCR and ligated into NotI and BamHI sites of Bluescript. The EcoRI fragment containing the acidic domain was then cloned into pGEX-4T-1. The rigor mutant of Myo3p was created by PCR-based mutagenesis of p1846 (a Myo3HA-containing plasmid obtained from Charlie Boone, Queens University, Kingston, Ontario) using the primers 5’-GCTTCTGTCTTACCCGCTCTAGACTCACC-3’ and 5’-GGTGAATCTAAGGCGGTTAAGACGAGACGGC-3’.

Strain Construction

The myo5ΔA Δmyo3 Δmyo5 strain was constructed by transforming a myo5ΔA-carrying plasmid (a COOH-terminal 50 amino acid [aa] deletion) into the double mutant background. The bee1ΔA strain was created by deletion of the COOH-terminal 33 codons of the Bee1 gene by homologous recombination. Deletion was confirmed by PCR and Western blot analysis. The bee1ΔA myo5ΔA Δmyo3 strain was made by crossing the bee1ΔA strain with the myo5ΔA Δmyo3 strain and subsequently selecting haploids with the desired genotype. All yeast strains used in this study are listed in Table I.

Isolation of Bee1p-Interacting Proteins

Extracts were prepared from RLY 650 cells by passing cells suspended in UB (50 mM Hepes, pH 7.5, 100 mM KCl, 3 mM MgCl2, 1 mM EGTA, 1 mM DTT, supplemented with protease inhibitors as described [Li et al., 1995]) through a French pressure cell at 2,300 psi. A high-speed supernatant was obtained by centrifugation at 250,000 g for 1 h. A ammonium sulfate was added to the resulting extract to bring it to 30% saturation (the protein A-tagged Bee1 is efficiently precipitated at this concentration). Precipitated protein was collected by centrifugation, resuspended in UB, and clarified. IgG-Sepharose beads (A mersham Pharmacal Biotech) were added to this solution and subsequently washed with UB plus 0.5% Tween 20, and then UB plus 1 M KCl. Protein was then eluted with 0.5 M acetic acid, pH 3.6. Bee1p-associated proteins were separated by one-dimensional SDS-PAGE and identified by high mass accuracy matrix-assisted laser desorption/ionization (MALDI) peptide mapping as described (Shevchenko et al., 1996a). In brief, protein bands visualized by staining with Coomassie were excised from the gel. Proteins were reduced by dithiothreitol, alkylated with iodoacetamide, and in-gel digested with trypsin (unmodified, sequencing grade; Boehringer Mannheim) at 37°C overnight (Shevchenko et al., 1996b). 0.5-μl aliquots of the supernatant
were withdrawn from the digest and analyzed on a R E F L E X M A L D I T O F mass spectrometer (Bruker Daltonics). Mass spectra were internally calibrated using known m/z of ions originating from the matrix and analysis products of trypsin. Database searches were performed using PeptideSearch 3.0 software developed in European Molecular Biology Laboratory. No limitations on protein molecular weights and species of origin were imposed. 18 (38) peptides were matched to the sequence of M yo3p (M yo5p) with the mass accuracy better than 75 ppm. These peptides covered 17 and 39% of the sequence of M yo3p and M yo5p, respectively.

**Protein Purification and Interaction Assays**

G S T -M yo3A contained the COOH-terminal 38 aa of M yo3p, and G S T -M yo5A and G S T -B ee1A contained the COOH-terminal 54 aa of each protein. Extracts were prepared from bacteria that were induced to express these proteins. Fusion proteins were purified on glutathione-agarose, washed extensively with PBS plus 0.5% T ween 20, eluted with reduced glutathione, and then equilibrated into U B . A rp2/3 complex was purified as described (Winter et al., 1999a). Purified A rp2/3 complex at 50 nM was incubated with fusion protein coupled beads, washed with U B plus 0.4% T ween 20, and then eluted into SDS-PAGE sample buffer. Immunoprecipitations were performed from 20 mg/ml, 250,000 g yeast extracts prepared by the liquid nitrogen grinding technique. In brief, anti-H A (monoclonal 12 CA 5 ascites; B A B C O ) or anti-myc (E van et al., 1985) coupled protein A beads were incubated with extract at 4°C for 1 h. After washing extensively with U B plus 0.4% T ween 20, proteins were eluted into SDS-PAGE sample buffer. For immunodepletions, concentrated extracts were treated as for immunoprecipitations, but the supernatant was removed for analysis.

**Actin Assembly Assays**

Small-budded cells and rhodamine-labeled actin were prepared as described (Li et al., 1995). The actin assembly and reconstitution assays in permeabilized cells was performed as described (Li et al., 1995; Lechler and Li, 1997). In brief, frozen small budded cells were permeabilized by incubation in 0.5 mg/ml saponin for 30 min, treated with 2 M urea for 25 min to inactivate actin assembly activity, and then incubated with concentrated extracts for 25 min. Extract was washed away and rhodamine-phalloidin (Molecular Probes) staining was performed as described (Pringle et al., 1989). Rhodamine-labeled actin (Kellogg et al., 1988) and phalloidin were imaged with a Zeiss A xiphot microscope with a H B 100 W/Z high pressure mercury lamp and a Zeiss 100× Plan N ef- fluor oil immersion objective. Image acquisition was carried out using Northern Exposure (Phase 3 Imaging Systems).

**Results**

**B e e l p Interacts with T y p e I M y o s i n s**

We demonstrated previously that B ee1p is required for actin assembly activity in permeabilized yeast cells. In part, B ee1p may function through activation of the A rp2/3 complex's nucleation activity. However, B ee1p contains several putative protein–protein interaction motifs, and appears to function with other proteins in the reconstitution of actin assembly (Lechler and Li, 1997). Therefore, an affinity chromatography strategy was used to identify B ee1p-interacting proteins. B ee1p was tagged at its C O O H terminus with four repeats of the I gG binding domain of protein A (B ee1p-Pr A ). Under the endogenous B E E 1 promoter, this construct rescued the growth defect of the Δ b ee1 mutation (data not shown). E xtracts were prepared from this strain, precipitated with ammonium sulfate to enrich for B ee1p, and then subjected to affinity chromatography on IgG-Sepharose beads. The ammonium sulfate treatment concentrates B ee1p, and possibly its binding partners as well, from the extract by ~28-fold. Bound proteins were eluted with acid, separated by SDS-PAGE, and individual protein bands were identified by mass spectrometry. Two proteins that bound specifically to B ee1p-Pr A and were present in approximately stoichiometric amounts were identified as M yo3p and M yo5p, the two type I myosins of budding yeast (F i g. 1 A ). Characterization of other proteins will be published elsewhere. Reciprocal immunoprecipitation was carried out to verify the B ee1p-myosin interaction in a strain that expresses H A -tagged M yo3p at an endogenous level. Although B ee1p was not detected in a control immunoprecipitation, the anti-H A immunoprecipitate specifically and reproducibly brought down B ee1p (F i g. 1 B ). However, only a small
fraction (<1%) of the total Bee1p was immunoprecipitated under these conditions, suggesting that the interaction is of low affinity. Concentration of the proteins through ammonium sulfate precipitation, as in Fig. 1 A, increases the level of interaction.

The yeast protein Vrp1p has been shown previously to interact with both Bee1p and Myo5p (Anderson et al., 1998; Naqvi et al., 1998). To determine whether the Bee1p–type I myosin interaction was mediated by Vrp1p, we performed immunoprecipitations of Myo3-HA from Δvrp1 cells. Consistently, we find that Bee1p is still present in the anti-HA immunoprecipitates, albeit at lower levels than in wild-type cells, suggesting that at least some fraction of the Bee1p interacts with type I myosins in a Vrp1p-independent manner (Fig. 1 B). These results, combined with the fact that both Bee1p and type I myosins localize to cortical patches (Goodson et al., 1996; Li, 1997) suggest that these proteins interact in vivo.

**Type I Myosins Are Required for the Reconstitution of Actin Assembly Sites**

Type I myosins have traditionally been thought of as molecular motors that transport cargo along actin filaments (Mooseker and Cheney, 1995). Therefore, their interaction with a protein involved in regulating actin dynamics was somewhat surprising. To determine whether the type I myosins are necessary for cortical actin polymerization. (A) Examples of positive (left panel, wild-type extract), and negative (right panel, myo3G132R mutant extract) results for the reconstitution of actin assembly sites in permeabilized yeast cells. A ssay conditions are described in Materials and Methods. (B) Concentrated extracts were prepared from various strains as indicated below the histograms and assayed for their ability to restore actin assembly activity to urea-treated permeabilized yeast cells. Quantitation of cells that assembled rhodamine actin into the bud was as described (Lechler and Li, 1997). For immunodepletion, anti-myc (mock depletion) or anti-HA coupled protein A beads were added to concentrated extracts prepared from a strain that expresses Myo3-HA from the Δmyo3 Δmyo5 background. After incubation, beads were pelleted and supernatants were removed for analysis. Gel inset above corresponding lanes of the histogram, shows depletion of Myo3-HA, but not Bee1p by the anti-HA beads. (C) Extracts from wild-type and Δmyo3 Δmyo5 cells were mixed at the indicated ratios and then assayed for their ability to restore actin assembly in urea-treated permeabilized cells. The complementation efficiency was quantified and plotted against the extract ratios. Each data point is an average of duplicate reactions.
myosins have a direct role in the production of actin filaments, extracts were prepared from single or double myosin knockout yeast strains and tested for their ability to reconstitute actin assembly in urea-inactivated permeabilized cells. Typical positive and negative results of this assay are shown in Fig. 2 A. Extracts prepared from ∆myo3 cells or ∆myo5 cells were able to restore actin assembly activity to the urea-inactivated permeabilized yeast cells to the same extent as wild-type extracts (Fig. 2 B). However, extracts from ∆myo3 ∆myo5 double mutant showed no more activity than addition of buffer alone. Therefore, the type I myosins are required for the actin assembly activity and appear to be functionally redundant in this assay as they are in vivo.

To demonstrate that the type I myosins are required for actin polymerization in a dose-dependent manner, wild-type and ∆myo3 ∆myo5 extracts were mixed at varying ratios to vary the concentration of the type I myosin at the same time as maintaining the concentration of other active components (Fig. 2 C). We find that as type I myosin concentration decreases, the ability to assemble actin also decreases. This closely resembles the results seen by dilution of wild-type extract with buffer alone (Lechler and Li, 1997), suggesting that the type I myosins are limiting factors in the wild-type extract.

To ensure that the lack of activity in the ∆myo3 ∆myo5 extract was not due to unhealthiness of the double mutant cells, we tested whether biochemical depletion of the type I myosins also led to a loss of ability to reconstitute actin assembly. To do so, a strain was generated that contained the HA-tagged Myo3p in the ∆myo3 ∆myo5 background. Therefore, Myo3-HA was the only source of type I myosins in this strain. The Myo3-HA rescued the growth defect of the double deletion strain (data not shown). Extracts were prepared from this strain and then mixed with protein A-Sepharose beads conjugated to either anti-HA or anti-myc (control) antibodies. Immunodepletion of the Myo3-HA is demonstrated in the immunoblot as shown in Fig. 2 B. Bee1p as well as the actin nucleator A rp2/3 complex (data not shown) were not significantly depleted from extracts by this treatment. The Myo3-depleted extract lacked the ability to restore actin assembly in urea-inactivated cells, whereas mock depletion had little effect (Fig. 2 B). These results support a direct involvement of the type I myosins in cortical actin assembly.

Motor Activity of Type I Myosins Is Necessary for Cortical Actin Polymerization

Type I myosins consist of an NH₂-terminal motor domain harboring the ATP and actin binding sites, followed by a tail domain that is thought to interact with cargos and other cytoskeletal proteins. We first took a pharmacological approach to determine whether the motor activity of myosins is required for actin polymerization. BDM is a low affinity inhibitor of myosins (Backx et al., 1994; Cramer and Mitchison, 1995). To separate the effect of the drug on the reconstitution of actin assembly sites from that on actin polymerization, BDM was either added during the incubation with the extract but washed out before the polymerization step, or was only present during the polymerization step. Addition of 20 mM BDM to extracts during reconstitution had no effect on actin assembly (data not shown), but when BDM was added with G-actin at the time of polymerization, actin assembly into the permeabilized cells was inhibited (Fig. 3). Similarly, addition of AMPPNP to extracts during the reconstitution had no effect on actin assembly activity (data not shown); however, when added with G-actin at the time of polymerization, it inhibited actin polymerization (Fig. 3). Because neither the same concentration of BDM nor AMPPNP blocked spontaneous assembly of pyrene-labeled actin (data not shown), these results suggest that myosin motor domain activity is not required during the reconstitution (e.g., to localize the proteins), but is required for actin polymerization at the cell cortex.

These pharmacological experiments support a role for myosins and ATP hydrolysis in actin assembly. However, care must be taken in interpreting these results, as the specificities of these inhibitors are broad. To further test the requirement of myosin motor activity in actin polymerization, Glycine132, within the P-loop required for nucleotide binding of Myo3p, was mutated to an arginine residue. Similar mutations in this loop of other myosins lead to an inability to bind nucleotide (Bjoseve and Anderson, 1990). Since nucleotide-free myosin binds tightly to actin filaments, these mutations are known as rigor mutations. The myo3G132R mutation results in a complete loss of function: it did not rescue the growth defect of the ∆myo3 ∆myo5 strain (data not shown). Extracts prepared from a strain carrying the rigor Myo3p as its sole source of type I myosins (i.e., in the ∆myo3 ∆myo5 background) were unable to restore actin assembly activity to the permeabilized cells (Fig. 3). This result further suggests that type I myosins require a functional motor domain in order to promote actin polymerization.

Type I Myosins Mediate Cdc42p Regulation of Cortical Actin Assembly

The distribution of actin patches in vivo as well as actin...
polymerization in the permeabilized cells are dependent on the small G T Pase Cdc42 (A dams et al., 1999; Li et al., 1995), but the mechanism by which Cdc42 regulates actin assembly in yeast was not understood. Because the motor activity of type I myosins, which appears to be required for cortical actin polymerization, is stimulated through phosphorylation by PAKs (Maruta and Korn, 1977; Wu et al., 1996; B rzeska et al., 1997, 1999), it is possible that the type I myosins mediate Cdc42 regulation of actin assembly through motor domain phosphorylation. Mutation of the conserved serine residue (ser357) to alanine in M yo3 leads to a complete loss of function, whereas mutation to aspartate has no phenotype (W u et al., 1997). To test the effects of these mutations on actin assembly, extracts were prepared from strains carrying the myo3S357A and M Y O 3 S357D mutants as their sole source of type I myosins. The myo3S357A mutant extract was unable to restore actin assembly in urea-treated cells, whereas the M Y O 3 S357D mutant extract reproducibly showed slightly higher levels of activity than wild-type extracts (Fig. 4 A).

To further establish a requirement of myosin I phosphorylation in cortical actin assembly, we reasoned that if this were true then the extract used for reconstitution of actin assembly should be sensitive to phosphatase treatment. In fact, incubation of extracts with alkaline phosphatase caused almost a complete loss of actin assembly activity (Fig. 4 A). Treatment of the M Y O 3 S357D extract with phosphatase, on the other hand, had very little effect on the activity of the extract. These data confirm the requirement for myosin I phosphorylation in actin assembly, and suggest that it is the only phosphorylation required for the reconstitution of actin assembly sites.

We showed previously that the actin assembly activity in permeabilized yeast cells was abolished by the cdc42-1 mutation (Li et al., 1995). If phosphorylation of the type I myosins is a key downstream event in the activation of actin assembly, then the M Y O 3 S357D mutation may rescue the actin assembly defect of cdc42-1 cells. To test this possibility, the M Y O 3 S357D and myo3S357A alleles were transformed into cdc42-1 cells, and actin polymerization was assayed in the permeabilized cells from the resulting strains. As shown in Fig. 4 B, permeabilized cdc42-1 cells expressing M Y O 3 S357D regained actin polymerization activity, whereas those expressing the myo3S357A allele were as defective as the untransformed cdc42-1 cells. This result supports the hypothesis that type I myosins are a key target of active Cdc42p in promoting actin polymerization.

**Involvement of Type I Myosins in the Activation of the Arp2/3 Complex**

A unique feature of the yeast type I myosins that is not shared by known type I myosins of other organisms is the presence of an acidic region at the C O O H-terminus. This domain shows significant similarity to the acidic C O O H-terminal tails of B ee1p and other members of W A S P family proteins. Over ~35 aa, the M yo3p and M yo5p tails are 45% conserved with the B ee1p tail motif (Fig. 5 A). This motif mediates the interaction of W A S P family proteins with the A rp2/3 complex and is essential for activation of the A rp2/3 complex's nucleation activity in vitro (M achesky and I nsall, 1998; Winter et al., 1999a). To determine if the myosins could interact with the A rp2/3 complex through the acidic motif, beads coupled to G S T fusions of M yo3p or M yo5p C O O H-terminal acidic fragments (G S T-M yo3A and G S T-M yo5A) were incubated with purified A rp2/3 complex, washed, and then examined for the presence of A rp2p. A rp2p was found associated with these fusion proteins to the same extent as with the B ee1p acidic domain (G S T-B ee1A) (Fig. 5 B). To determine whether the interaction between the type I myosins and A rp2/3 complex occurs in vivo, we immunoprecipitated H A -tagged M yo3p expressed at an endogenous level from cell extracts, and blotted with an antibody against A rp2p. A rp2p was detected in M yo3-H A immunoprecipitates but not in control immunoprecipitates (Fig. 5 C). As we have found previously with B ee1p, this interaction is of
low affinity, and <1% of total Arp2p is immunoprecipitated with the type I myosins (data not shown). The COOH-terminal domain of Myo3p was required for this interaction, as a deletion mutant lacking the COOH-terminal 34 aa no longer immunoprecipitated Arp2p (Fig. 5 C). This result, together with the observation that Myo3p, Myo5p, A rp2p, and A rp3p all localize to cortical actin patches, suggest that the type I myosins and the Arp2/3 complex interact in vivo (Goodson et al., 1996; Moreau et al., 1996; Winter et al., 1997).

We showed previously that deletion of the Arp2/3-interacting domain of Bee1p does not cause severe defects in cortical actin organization, as opposed to disruption of the entire BEE1 gene or subunits of the Arp2/3 complex (Winter et al., 1999a). To determine whether this lack of phenotype was due to redundant functions of the homologous domains in the type I myosins, strains were created that contained deletions of these domains in combination. The growth defect of a Δmyo3 Δmyo5 double mutant is fully rescued by an allele of Myo3p in which the COOH-terminal 34 aa was deleted (Δmyo3Δ34) (Fig. 6 A). However, although neither the bee1ΔA mutation alone nor the myo5ΔA truncation in the Δmyo3 Δmyo5 double mutant background showed any severe phenotypes, a combination of the two in the Δmyo3 Δmyo5 double mutant background had a clear synthetic effect: the mutant cells grew almost as poorly as the Δmyo3 null cells at 30°C and did not grow at 37°C. The double mutant cells also show severe defects in actin organization and accumulate abnormal F-actin aggregates, characteristic of null alleles of BEE1 and genes encoding subunits of the Arp2/3 complex (Fig. 6 B) (Li, 1997; Winter et al., 1999b). These data suggest that the homologous COOH-terminal domains of Bee1p and type I myosins have a redundant but important role in regulation of the actin in vivo.

This synthetic effect is also evident in vitro. Extracts were prepared from the bee1ΔA, the myo5ΔA Δmyo3, and the bee1ΔA myo5ΔA Δmyo3 strains and tested for their ability to reconstitute actin polymerization in the urea-inactivated permeabilized cells. Whereas the actin assembly activity was intact in the former two extracts, an extract from the strain lacking both Bee1p and Myo5p COOH-terminal acidic domains (in the Δmyo3 Δmyo5 double mutant background) had essentially no activity (Fig. 7 A). These results suggest that the COOH-terminal domains of Bee1p and Myo5p share a redundant function both in vivo and in vitro. Finally, to address how the concentration of these activation domains affects actin assembly activity, we titrated wild-type extract with the bee1ΔA myo5ΔA Δmyo3 extract (Fig. 7 B). A ctin assembly decreases at low levels of the activation domain; however, actin assembly is less sensitive to loss of activation domains than to loss of type I myosins (compare Fig. 2 C and Fig. 7 B).

Discussion

Type I myosins are a highly conserved family of molecular motors, first identified almost 30 years ago (for review see Mooseker and Cheney, 1995). In spite of this, precise in vivo function of these myosins have not been defined. Genetic and biochemical evidence in Acanthamoeba, Dictyostelium, and yeast have implicated these proteins in actin-based processes such as cell motility, phagocytosis, endocytosis, and contractile activity (Mooseker and Cheney, 1995). Type I myosins have been hypothesized to transport membranous vesicles or to provide contractile force on actin filaments in order to function in these processes. Here we have presented evidence that type I myosins are directly involved in the promotion of actin assembly at the cell cortex.

Myo3p and Myo5p physically interact with Bee1p, the yeast WA5P homologue. This interaction appears to be of
low affinity, as only a small fraction coimmunoprecipitates. However, we consistently find that increasing extract concentration increases the amount of bound proteins. In the case where extracts were first precipitated with 30% ammonium sulfate, we achieved a 28-fold concentration over the starting extract, leading to an almost stoichiometric interaction between the proteins. This suggests that with the high in vivo concentrations of these proteins in cortical patches, the majority of these proteins may be in complex. The interaction between Bee1p and the myosins is likely to occur through interaction of the SH3 domain of the myosins and the proline-rich repeats of Bee1p (M. Evangelista and C. Boone, personal communication). The only other identified ligand for the SH3 domain of type I myosins is Acan125, a protein purified from Acanthamoeba which shows no homology to Bee1p (Xu et al., 1998).

Figure 6. The type I myosin and Bee1p acidic tails have redundant functions. (A) Equal concentration of cells from strains as indicated were spotted onto yeast extract, peptone, dextrose (YPD) plates, grown for 36 h, and then photographed. myo5ΔA Δmyo3: a strain expressing myo5ΔA in the Δmyo3 Δmyo5 double mutant background; bee1ΔA myo5ΔA Δmyo3: a strain expressing bee1ΔA and myo5ΔA in the Δmyo3 Δmyo5 double mutant background. (B) Rhodamine-phalloidin staining (upper panels) and differential interference contrast (DIC) images (lower panels) of wild-type and bee1ΔA myo5ΔA Δmyo3 cells grown at room temperature.

Figure 7. Bee1p and type I myosin acidic tails function redundantly in actin assembly. (A) Concentrated extracts were prepared from the strains as indicated and tested for their ability to restore actin assembly activity of urea-treated permeabilized yeast cells. (B) Extracts from wild-type and bee1ΔA myo5ΔA Δmyo3 cells were mixed at the indicated ratios on the horizontal axis and then assayed for their ability to restore actin assembly in urea-treated permeabilized cells. The complementation efficiency was quantified and plotted against the extract ratios. Each data point is an average of duplicate reactions.
Interestingly, the Bee1p binding protein, Vrp1p, also interacts with the SH3 domain of type I myosins (Anderson et al., 1998; Naqvi et al., 1998). Since the SH3 domain of the yeast type I myosins was shown to be important for their localization to actin patches (Anderson et al., 1998), it is possible that Bee1p and Vrp1p function redundantly in targeting the myosins to regions of actin assembly. Another possibility is that Bee1p and Vrp1p are cargo for the myosins and are transported to the barbed ends of filaments by these molecular motors (see below).

Regardless, since all these proteins have mammalian counterparts (Vrp1p is a homologue of the mammalian WASP-interacting protein [WIP] protein [Ramesh et al., 1997]), it would be interesting to test if their complex formation is also conserved.

The type I myosins also interact with the Arp2/3 complex. The COOH-terminal tails of Myo3p and Myo5p show significant homology to the COOH-terminal tails of Bee1p and other WA SP family proteins. These acidic tails are actually more similar to WA SP and N-WA SP than they are to Bee1p, as they have a large net negative charge, as opposed to Bee1p which has a more neutral charge. These motifs mediate a direct physical interaction with the Arp2/3 complex. This interaction is likely to occur in vivo, as a Arp2/3 complex can be immunoprecipitated with the type I myosins, an association that is dependent upon the COOH-terminal tail of the myosins. There are several possible functions for this interaction. First, it could be involved in localization of either the myosins or the Arp2/3 complex. This is unlikely, as a Arp2/3 complex localization is not affected by deletion of the myosin acidic tail motif (data not shown). A second, deletion of the myosin acidic tails causes no obvious defects in the presence of wild-type Bee1p, suggesting that myosin localization is unlikely to be abolished. A third possible function for the myosin–Arp2/3 interaction could be to activate the nucleation activity of the Arp2/3 complex. This possibility is supported by three findings. First, homologous motifs in Bee1p and WA SP proteins have been shown to be required for the activation of the Arp2/3 complex (machesky et al., 1999; Rohatgi et al., 1999; Winter et al., 1999a; Y arar et al., 1999). Second, we found a specific synthetic interaction due to loss of the Bee1p and myosin I acidic motifs: loss of the Arp2/3-activating acidic domain of either Bee1p or the type I myosins does not cause a dramatic phenotype; however, in combination, these deletions lead to drastic growth and actin organization defects. Third, by using an in vitro reconstitution assay, we showed that loss of the acidic motifs of Bee1p and type I myosins causes a defect in actin assembly activity. These data support redundant functions of the myosin and Bee1p acidic domains in activation of the Arp2/3 complex.

It was surprising to us that an involvement in actin polymerization is not limited to the tail domain of type I myosin, but that motor activity is also required. The requirement for myosin motor activity in actin polymerization may be explained by one of the following models. First, the type I myosins may function by transporting nucleation or elongation machinery to the barbed ends of existing filaments. If the cargo is a WA SP family member or the Arp2/3 complex, this would ensure that the nucleation machinery remains near the barbed end of filaments, promoting the generation of dendritic actin filaments at the membrane cortex. Alternatively, if the cargo is an elongation machinery (Zigmond et al., 1998), it could serve to maintain this machinery at the growing barbed end of actin filaments. Type I myosins may also function in cortical filament assembly as a molecular ratchet. In this model, type I myosins associate with the membrane through the lipid-binding domain within their tails (Adams and Pollard, 1989); movement of the myosin head along actin towards the barbed end could then generate a membrane protrusion, allowing addition of G-actin onto the barbed end. This would explain the requirement for myosin motor activity during polymerization. In filopodia, it has been proposed that such a function may be important for coupling polymerization and leading edge protrusion (Sheetz et al., 1992). One caveat is that all of these models imply processivity of the myosin motor. In vitro experiments suggested that individual myosin I molecules are unlikely to remain bound to F-actin long enough to translocate through any significant distance (O stap and Pollard, 1996).

However, other experiments have shown that myosin I can transport vesicles or F-actin filaments (Adams and Pollard, 1986; Z ot et al., 1992), possibly due to a second F-actin binding site in the myosin I tail that can cross-link actin filaments (Fujisaki et al., 1985). A n alternative possibility is that the type I myosins do not function like conventional motors in promoting actin assembly. For example, members of the K I kinesin family of microtubule motors have been shown to use their microtubule-dependent ATPase activity to catalyze the depolymerization of microtubules (Desai et al., 1999).

The mammalian N-WA SP protein has been shown to mediate Cdc42-dependent actin polymerization by interacting directly with Cdc42 (Maki et al., 1998a; R ohatgi et al., 1999). However, WA SP-family proteins such as Bee1p and WA V E , do not contain the Cdc42-binding motif (Li, 19997; Maki et al., 1998b). In yeast, PA K s are known Cdc42 effectors. The involvement of yeast PA K s in actin polymerization was first suggested by the result that overexpression of Ste20p, a yeast PA K , suppresses both the polymerization and actin assembly defects of cdc42-1 mutant (E by et al., 1998). W e have shown here that a mutation in Myo3p mimicking constitutive phosphorylation of the site known to be acted upon by PA K s rescues the actin assembly defects in cdc42-1 mutant cells. This finding suggests that Myo3p and probably also Myo5p are major targets of Cdc42/PA K in the regulation of actin assembly at the cell cortex. A function in mediating polarized actin assembly may explain the phenotype of myosin I mutant Dicyostelium cells that exhibit defects in maintaining direction of movement in chemotactic streaming assays (J ung et al., 1996; Titus et al., 1993). Since the PA K phosphorylation site is conserved in amoeba type I myosins, but not in those of higher cells, it is possible that this pathway of actin regulation by small GTPases only operates in protozoan organisms. However, the mammalian type VI myosins, which were recently shown to move toward the pointed ends of actin filaments (Wells et al., 1999), have the same PA K phosphorylation sites (B uss et al., 1998). It will be interesting to define the role of these myosins in actin polymerization and actin-based motility.


