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

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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 spectrometric analysis. The yeast type I myosins, Myo3p and Myo5p, 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 triphatase, 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.

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),1 downstream targets of Rho family small GTPases (M aruta and K orn, 1997; 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 (W u 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, Myo3p and Myo5p, which are functionally redundant in vivo (Goodson et al., 1996). These myosins consist of an NH2-terminal motor domain with F-actin binding, contractility, and membrane proteins (MYO3 and MYO5), 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 (W u et al., 1997). Phosphorylation is required for function in vivo, as a nonphosphorylatable Myo3p mu-
tand does not rescue the growth defect of the double myo-
sin I-null (Wu et al., 1997). Ste20p and Cla4p are effectors
of the small GTPase Cdc42p (for review see Daniels and
Bokoch, 1999), which is required for polarized cell growth
during bud formation (Adams et al., 1990). Thus, yeast
provides an excellent system for elucidating the in vivo
function and regulation of type I myosins.

Cdc42 is required for cell polarity and actin polymeriza-
tion in a number of systems. A requirement for Cdc42 ac-
tivity in actin assembly has been shown in permeabilized
yeast cells, and Acanthamoeba, 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
have been the subject of recent research. In both Dicty-
stelium and X enopus egg extracts, the actin-related protein
(A rp) 2/3 complex is required for Cdc42-induced actin ac-
sembly (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 flex-
neri (Welch et al., 1997; G eile et al., 1999). The nucleation
activity of purified A rp2/3 complex is low but can be
greatly stimulated by the Listeria A cT protein (Welch
et al., 1998), and by members of the Wiskott-Aldrich syn-
drome protein (WA SP) family, including WA SP, neuronal
(N)-WA SP, WA VE SCA R1, and B ee1p/Las17p (M a-
chesky et al., 1999; Rohatgi et al., 1999; Winter et al.,
1999a; Y arar 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 ac-
tivate the nucleation activity of the A rp2/3 complex is de-
pendent upon binding to Cdc42p (Rohatgi et al., 1999).
It is believed that Cdc42 binding induces a conformational
change in N-WA SP, exposing its A rp2/3 activation do-
main. WA VE and B ee1p lack the Cdc42-interacting do-
main, 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 associ-
ated with cortical patches in permeabilized yeast cells was
first eliminated by treatment with 2 M urea and reconsti-
tuted 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 B ee1p, 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 A rp2/3-
binding domain of B ee1p has little effect on actin assem-
by and cell growth, suggesting that B ee1p is unlikely to be
the sole activator of the A rp2/3 complex (Winter et al.,
1999a). In this study, we first identified the yeast type I
myosins as B ee1p-interacting proteins that are also re-
quired 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 re-
dundantly with B ee1p in the activation of the A rp2/3
complex.

Materials and Methods

Plasmid Construction

The M yo5A C-hemagglutinin (HA) construct was prepared by PCR of the
promoter (–284) and the first 1169 codons of M yo5 O using genomic DNA
and the primers 5′-GGCGGGCGCCGGGGGTGTTTTAATCTCG-
TGCG-3′ and 5′-GGCCGGGCAATCCTTGACGTACGGCGG-3′. The prod-
guct was digested with NotI and ligated into PRL129, an HA -tagging vector from
PR306. The M yo5A C-HA construct was generated by PCR using the primers 5′-GGC-
GCGGGCGCCGGCCCTGGGAAGAGCCTTTACCGC-3′ and 5′-GGCC-
GGACATTCGTGAGGGACGTATTACAGCAGCGCGG-3′. This prod-
guct was digested with NotI and EcoRI and ligated into PRL129. The glutathione
S-transferase (GST) -M yo3A -expressing plasmid was made by PCR of
the acidic domain of M yo5 O using the primers 5′-GGCGGGGAATT-
CTCGTGGGAGCCTAAGGCGG-3′ and 5′-GGCCGGGAATT-
CCTGAGTATTACACGTATTGCCG-3′, digesting the product with
EcoRI, and ligating into the EcoRI site of pGEX4T-1. The GST-M yo5A
construct was created by subcloning of the M yo5p acidic domain. Full-
length M yo5 O was subjected to PCR and ligated into NotI, BamHI sites of
Bluescript. The EcoRI fragment containing the acidic domain was then
digested into pGEX4T-1. The rigor mutant of M yo3p was created by PCR-
based mutagenesis of pB109 (a M yo3A-HA-containing plasmid obtained
from Charlie Boone, Queens University, Kingston, Ontario) using the
primers 5′-GCTTCTGCTTACCGGCTACTGATCATTCCC-3′ and 5′-
GGTAGCTATAGACGGGTGAGAAAGACAGACG-3′.

Strain Construction

The m yo5A A m yo3 m yo5 strain was constructed by transforming a
m yo5A -carrying plasmid (a COOH-terminal 50 amino acid [aa] dele-
tion) into the double mutant background. The b ee1A strain was created
by deletion of the COOH-terminal 33 codons of the B ee1 gene by ho-
mologous recombination. Deletion was confirmed by PCR and Western
blot analysis. The b ee1A m yo5A A m yo3 strain was made by crossing the
b ee1A strain with the m yo5A A m yo3 strain and subse-
sequently selecting haploids with the desired genotype. All yeast strains
used in this study are listed in Table I.

Isolation of Bee1p-Interacting Proteins

E xtracted were prepared from RLY 650 cells by passing cells suspended in
UB (50 mM H epes, 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 superna-
tant was obtained by centrifugation at 250,000 × g for 1 h. A ammonium sul-
fate was added to the resulting extract to bring it to 30% saturation (the
protein A-tagged B ee1 is efficiently precipitated at this concentration).
Precipitated protein was collected by centrifugation, resuspended in
UB, and clarified. IG G -Sepharose beads (A mersham Pharmacia B iotech)
were added to this solution and subsequently washed with UB plus 0.5%
T ween 20, and then UB plus 1 M KCl. Protein was then eluted with 0.5 M
acetic acid, pH 3.6. B ee1p-associated proteins were separated by one-
dimensional SDS-PAGE and identified by high mass accuracy matrix-
assisted laser desorption/ionization (MALDI) peptide mapping as de-
scribed (Shevchenko et al., 1996a). In brief, protein bands visualized by
staining with C0omassie were excised from the gel. Proteins were reduced
by dithiothreitol, alkylated with iodoacetamide, and in-gel digested with
trypsin (unmodified, sequencing grade; B oehringer M annheim) at 37°C
overnight (Shevchenko et al., 1996b). 0.5-μl aliquots of the supernatant
purified as described (Winter et al., 1999a). Purified Arp2/3 complex at 50 mM reduced glutathione, and then equilibrated into UB. Arp2/3 complex was treated 17 and 39% of the sequence of Myo3p and Myo5p, respectively. These peptides covered 17 and 39% of the sequence of Myo3p and Myo5p, respectively.

**Fluorescent Microscopy**

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 x100 oil immersion objective. Image acquisition was carried out using Northern Exposure (Phase 3 Imaging Systems).

**Results**

**Bee1p Interacts with Type I Myosins**

We demonstrated previously that Bee1p is required for actin assembly activity in permeabilized yeast cells. In part, Bee1p may function through activation of the Arp2/3 complex’s nucleation activity. However, Bee1p 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 Bee1p-interacting proteins. Bee1p was tagged at its COOH terminus with four repeats of the IgG binding domain of protein A (Bee1p-PrA). Under the endogenous myosin I regulative phenotype, this construct rescued the growth defect of the bee1Δ mutant (data not shown). E xtracts were prepared from this strain, precipitated with ammonium sulfate to enrich for Bee1p, and then subjected to affinity chromatography on IgG-Sepharose beads. The amnonium sulfatetreatment concentrates Bee1p, 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 Bee1p-PrA were identified as Myo3p and Myo5p, the two type I myosins of budding yeast (Fig. 1A). Characterization of other proteins will be published elsewhere. Reciprocal immunoprecipitation was carried out to verify the Bee1p-myosin interaction in a strain that expresses HA-tagged Myo3p at an endogenous level. Although Bee1p was not detected in a control immunoprecipitate, the anti-HA immunoprecipitate specifically and reproducibly brought down Bee1p (Fig. 1B). However, only a small

| Name | Genotype | Source |
|------|----------|--------|
| RLY1 | MATα his3Δ200 leu2-3 lys2-801 ura3-52 | Drubin lab |
| RLY171 | MATα leu2-3 ura3-52 cdh42-1 | Drubin lab |
| RLY146 | MATα ade2 ade3Δ200 leu2-3 trp1-1 ura3-52 bar1 Δmyo3::His3 Δmyo5::TRP1 pMYO3-HA (p1846) | This work |
| RLY1458 | MATα his3Δ200 leu2-3 lys2-801 ura3-52 Δbee1::LEU12 pBE1-PrA (pTL84) | Winter et al., 1999 |
| RLY1602 | MATα ade2 can1 his3Δ200 leu2-3 trp1-1 ura3-52 Δmyo3::His3 Δmyo5::TRP1 pmyo3-S357A (pVL97) | This work |
| RLY1603 | MATα ade2 can1 his3Δ200 leu2-3 trp1-1 ura3-52 Δmyo3::His5 S357D (pVL98) | This work |
| RLY1804 | MATα ade2 can1 his3Δ200 leu2-3 trp1-1 ura3-52 Δmyo3::His3 Δmyo5::TRP1 pmyo5ΔC-HA (pTL88) | This work |
| RLY1805 | MATα ade2 can1 his3Δ200 leu2-3 trp1-1 ura3-52 Δmyo3::His3 Δmyo5::TRP1 pmyo3ΔC-HA (pTL91) | This work |
| RLY1817 | MATα his3Δ200 leu2-3 lys2-801 trp1-1 ura3-52 Δmyo5::TRP1 | This work |
| RLY1819 | MATα his3Δ200 leu2-3 lys2-801 trp1-1 ura3-52 Δmyo5::TRP1 | This work |
| RLY822 | MATα his3Δ200 leu2-3 lys2-801 trp1-1 ura3-52 Δmyo5::His3 Δmyo5::TRP1 | This work |
| RLY865 | MATα his3Δ200 leu2-3 lys2-801 trp1-1 ura3-52 Δmyo5::His3 Δmyo5::TRP1 pmyo3-G132R-HA (pTL103) | This work |
| RLY871 | MATα his3Δ200 leu2-3 lys2-801 trp1-1 ura3-52 Δmyo5::His3 Δmyo5::TRP1 pmyo3::URA3::myo5C::bee1ΔC::LEU2 | This work |
| RLY889 | MATα leu2-3 ura3-52 cdh42-1 pmyo3-S357A (pVL97) | This work |
| RLY890 | MATα leu2-3 ura3-52 cdh42-1 pMYO3-S357D (pVL98) | This work |
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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. 1A, 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. 1B). 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, myo3Δ132R mutant extract) results for the reconstitution of actin assembly sites in permeabilized yeast cells. Assay 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 in 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 Δmyo3 cells or Δmyo5 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 Δmyo3 Δmyo5 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 Δmyo3 Δmyo5 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 Δmyo3 Δmyo5 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 Δmyo3 Δmyo5 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. Becllp as well as the actin nucleator Arp2/3 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 dur-
polymerization in the permeabilized cells are dependent on the small GTPase Cdc42 (Adams et al., 1990; 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., 1997), 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 Myo3 leads to a complete loss of function, whereas mutation to aspartate has no phenotype (Wu et al., 1997). To test the effects of these mutations on actin assembly, extracts were prepared from strains carrying the myo3S357A and MYO3S357D alleles as their sole source of type I myosins. The myo3S357A mutant extract was unable to restore actin assembly in urea-treated cells, whereas the MYO3S357D 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 MYO3S357D 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 MYO3S357D mutation may rescue the actin assembly defect of cdc42-1 cells. To test this possibility, the MYO3S357D 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 MYO3S357D 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 COOH-terminus. This domain shows significant similarity to the acidic COOH-terminal tails of Bee1p and other members of WASP family proteins. Over ~35 aa, the Myo3p and Myo5p tails are 45% conserved with the Bee1p tail motif (Fig. 5 A). This motif mediates the interaction of WASP family proteins with the Arp2/3 complex and is essential for activation of the Arp2/3 complex's nucleation activity in vitro (Machesky and Insall, 1998; Winter et al., 1999a). To determine if the myosins could interact with the Arp2/3 complex through the acidic motif, beads coupled to GST fusions of Myo3p or Myo5p COOH-terminal acidic fragments (GST-Myo3A and GST-Myo5A) were incubated with purified Arp2/3 complex, washed, and then examined for the presence of Arp2p. A rp2p was found associated with these fusion proteins to the same extent as with the Bee1p acidic domain (GST-Bee1A) (Fig. 5 B). To determine whether the interaction between the type I myosins and Arp2/3 complex occurs in vivo, we immunoprecipitated HA-tagged Myo3p expressed at an endogenous level from cell extracts, and blotted with an antibody against Arp2p. A rp2p was detected in Myo3-HA immunoprecipitates but not in control immunoprecipitates (Fig. 5 C). As we have found previously with Bee1p, 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 Arp2p/3 complex interact in vivo (Goodson et al., 1996; Moreau et al., 1996; Winter et al., 1997).

We showed previously that deletion of the A rp2p/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 A rp2p/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 with deletion of the COOH-terminal A rp2p/3-interacting domain (34 aa) was deleted (Δmyo3ΔA) (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 null strains. 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 in vivo.

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 actin assembly at the cell cortex.

Myo3p and Myo5p physically interact with Bee1p, the yeast WASP 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., 1997).

**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. Reconstitution procedure and quantitation of the results were performed as described (Lechler and Li, 1997). (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 WASP family proteins. These acidic tails are actually more similar to WA SP and N-WASp 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 loss, 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 other 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 WASP proteins have been shown to be required for the activation of the Arp2/3 complex (Maivesky et al., 1999; Rohatgi et al., 1999; Winter et al., 1999a; Yarar 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 WASP 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 (Zigmund 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 proccessivity 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 (Ostap and Pollard, 1996). However, other experiments have shown that myosin I can transport vesicles or F-actin filaments (Adams and Pollard, 1986; Zot 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). An alternative possibility is that the type I myosins do not function like conventional motors in promoting actin assembly. For example, members of the K in 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-WASp protein has been shown to mediate Cdc42-dependent actin polymerization by interacting directly with Cdc42 (Miki et al., 1998b; Rohatgi et al., 1999). However, WASP-family proteins such as Bee1p and Vrp1p do not contain the Cdc42-binding motif (Li, 1997; Miki et al., 1998b). In yeast, PAKs are known Cdc42 effectors. The involvement of yeast PAKs in actin polymerization was first suggested by the result that overexpression of Ste20p, a yeast PAK, suppresses both the polarization and actin assembly defects of cdc42-1 mutant (Eby et al., 1998). We have shown here that a mutation in Myo3p mimicking constitutive phosphorylation of the site known to be acted upon by PAKs rescues the actin assembly defects in cdc42-1 mutant cells. This finding suggests that Myo3p and probably also Myo5p are major targets of Cdc42p/PAK 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 Dictyostelium cells that exhibit defects in maintaining direction of movement in chemotactic streaming assays (Jung et al., 1996; Titus et al., 1993). Since the PAK 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 towards the pointed ends of actin filaments (Wells et al., 1999), have the same PAK phosphorylation sites (Buss et al., 1998). It will be interesting to define the role of these myosins in actin polymerization and actin-based motility.
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