A Role for Myosin-I in Actin Assembly through Interactions with Vrp1p, Bee1p, and the Arp2/3 Complex

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Abstract. Type I myosins are highly conserved actin-based molecular motors that localize to the actin-rich cortex and participate in motility functions such as endocytosis, polarized morphogenesis, and cell migration. The COOH-terminal tail of yeast myosin-I proteins, Myo3p and Myo5p, contains an Src homology domain 3 (SH3) followed by an acidic domain. The myosin-I SH3 domain interacted with both Bee1p and Vrp1p, yeast homologues of human WASP and WIP, adapter proteins that link actin assembly and signaling molecules. The myosin-I acidic domain interacted with Arp2/3 complex subunits, Arc40p and Arc19p, and showed both sequence similarity and genetic redundancy with the COOH-terminal acidic domain of Bee1p (Las17p), which controls Arp2/3-mediated actin nucleation. These findings suggest that myosin-I proteins may participate in a diverse set of motility functions through a role in actin assembly.

Key words: yeast • myosin-I • Arp2/3 • actin assembly • WASP

Introduction

Cortical actin polymerization underlies cell movement, polarization, morphogenesis, and cytokinesis (Drubin and Nelson, 1996; Mitchison and Cramer, 1996). Temporal and spatial coordination of these events depends upon adapter proteins, such as members of the WASP (Wiskott-Aldrich syndrome protein), Enabled, and Formin families, which localize to the cell surface and link signaling pathways to actin assembly proteins (Gertler et al., 1996; Symons et al., 1996; Frazier and Field, 1997; Wasserman, 1998; Ramesh et al., 1999; Bi and Zigmond, 1999; Machesky and Insall, 1999). The dynamic nature of actin polymerization makes it likely that actin assembly machinery associates with actin-based molecular motors. Type I unconventional myosins are attractive candidates for motors involved in actin assembly due to their high conservation and participation in a variety of cortical motility processes (Baines et al., 1992; Wagner et al., 1992; McGoldrick et al., 1995; Novak et al., 1995; Jung et al., 1996; Titus, 1998; Mermall et al., 1998), however their roles at a molecular level have remained uncertain. Here we establish that budding yeast *Saccharomyces cerevisiae* type I myosins functionally associate with both Bee1p (Las17p), a WASP-like adapter protein, and with components of the Arp2/3 actin nucleation complex, suggesting myosin-I proteins participate in the assembly of cortical actin filaments.

Yeast Bee1p (Winter et al., 1999a) and other members of the WASP family (Machesky and Insall, 1998; Egile et al., 1999; Machesky et al., 1999; Rohatgi et al., 1999; Suetsugu et al., 1999) bind and activate the Arp2/3 complex. This complex appears to be a critical component of the actin cytoskeleton in all eukaryotes. It consists of seven proteins including two actin-related proteins, Arp2p and Arp3p, and localizes to sites of dynamic filamentous actin such as the leading edges of locomoting cells, the actin comet tails of the intracellular pathogen, *Listeria monocytogenes*, and motile cortical actin patches in yeast (McCollum et al., 1996; Moreau et al., 1996, 1997; Mullins et al., 1997; Welch et al., 1997, 1998; Winter et al., 1997, 1999b; Beckerle, 1998; Machesky, 1999; Weiner et al., 1999). The Arp2/3 complex nucleates and caps the pointed end of an actin filament, which then elongates at the fast-growing barbed end, and binds to the side of filaments leading to cross-links and branches (Mullins et al., 1998a,b; Svitkina and Borisy, 1999). These activities enable the construction of filament networks, such as those observed in the lamellipodia of migrating cells and the actin clouds around *Listeria*. In yeast, the genes encoding the subunits of the Arp2/3 complex are required for endocytosis and the assembly, polarization, and movement of cortical actin patches (McCollum et al., 1996; Moreau et al., 1996, 1997; Winter et al., 1997, 1999b).

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Myosin-I proteins are single-headed nonfilamentous motors consisting of a heavy chain and one or more calmodulin or calmodulin-like light chains. The NH_2-terminal head domain of the heavy chain displays actin-stimulated Mg-ATPase activity and generates a mechanochemical force (Albanesi et al., 1985; Adams and Pollard, 1986; Zot et al., 1992; Ostap and Pollard, 1996). Two functionally redundant genes, *MYO3* and *MYO5*, code for the heavy chain of yeast type I myosins. Deletion of both genes compromises several actin-dependent functions, leading to a severe growth defect or lethality depending upon the genetic background (Goodson et al., 1996; Geli and Riezman, 1996). In contrast, the single mutants appear relatively normal, although *myo5Δ* cells are particularly temperature-sensitive for endocytosis (Geli and Riezman, 1996; Geli et al., 1998). Myo3p and Myo5p localize to cortical patches that are enriched at sites of polarized growth and often overlap with actin patches (Goodson et al., 1996; Anderson et al., 1998). As observed for the type I myosins of *Dictyostelium* and *Acanthamoeba* (Lee et al., 1996; Brzeska et al., 1996; Novak and Titus, 1998), Myo3p and Myo5p are activated through phosphorylation of a single site in the head domain by PAK-like kinases, Ste20p and Cla4p (Wu et al., 1996, 1997). The tails of unconventional myosins are expected to participate in molecular interactions that specify the role of the motor domain. Yeast type I myosin tails contain a potential phospholipid-binding domain, followed by an SH3 domain and a COOH-terminal acidic domain (Goodson and Spudich, 1995; Goodson et al., 1996; Geli and Riezman, 1996).

Materials and Methods

Yeast Strains

Unless stated otherwise, yeast strains were derived from W3031A (*Mat***a** *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100*) or W3031B (*Matα ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100*) carrying *myo3Δ::HIS3* and *myo5Δ::TRP1* alleles (Anderson et al., 1998). Gene fusions leading to a COOH-terminal tag containing 3 copies of the hemagglutinin (HA)[1] epitope (3HA) or 13 copies of the Myc epitope were constructed by PCR-based integration (Longtine et al., 1998). For coimmunoprecipitation experiments involving Myo3p, W3031A strains expressing Bee1p-HA, Y1836 (*myo3Δ::HIS3 BEE1::3HA-TRP1*), and Vrp1p-HA, Y1447 (*myo3Δ::HIS3 myo5Δ::TRP1 VRP1::3HA-kanMX6*) and W3031B strains expressing Arc40p-HA, Y1790 (*myo3Δ::HIS3 ARC40::3HA-kanMX6*), and Arc19p-HA, Y1789 (*myo3Δ::HIS3 ARC19::3HA-kanMX6*), were transformed with pRS315-derived plasmids encoding Myo3p (pVL96), Myo3p(SH3-W1157S) (p1754), or Myo3pΔAD (p2921). The Vrp1p and Bee1p coimmunoprecipitation experiment was performed with a W3031A strain expressing both Bee1p-HA and Vrp1p-Myc, Y2314 (*BEE1::3HA-TRP1 VRP1::13Myc-kanMX6*), and a W3031A control strain expressing Bee1p-HA, Y2312 (*BEE1::3HA-TRP1*). In contrast to the corresponding gene deletion mutants, the *BEE1::3HA-TRP1, VRP1::3HA-kanMX6, VRP1::13Myc-kanMX6, ARC40::3HA-kanMX6*, and *ARC19::3HA-kanMX6* alleles did not perturb cell growth rate or polarity of the actin cytoskeleton, indicating they lead to functional HA-tagged proteins. For testing the genetic redundancy of the myosin-I acidic domain and the Bee1p acidic domain, we constructed the W3031A derivatives Y1899 (*myo3Δ::HIS3 myo5Δ::TRP1 bee1ΔAD::3HA-kanMX6 GAL1-MYO3*) and Y1900 (*myo3Δ::HIS3 myo5Δ::TRP1 GAL1-MYO3*). Y1899 contains the *bee1ΔAD::3HA-kanMX6* allele, which was generated by PCR-based integration of a *3HA-kanMX6* cassette with primers (5′-AGCTTCAATTAGAGGGGCAGGTGGCATAGGCGCTTTGAGAAAAGTTGACAAATCGCGGATCCCCGGGTTAATTAA-3′) and (5′-GTACATAAAATTACATATTTTCTATAACAGTAGTTTCATCTTTGTTTGCATTCAGAATTCGAGCTCGTTTAAAC-3′) and resulted in deletion of 63 COOH-terminal residues of Bee1p. The *bee1ΔAD::3HA-kanMX6* allele did not perturb growth or actin organization, but produced a truncated form of Bee1p as observed by immunoblot analysis (data not shown). Both Y1899 and Y1900 carry a *LEU2*-based plasmid, pBK28, encoding *GAL1-MYO3*. To generate Y2136 (*bee1ΔAD myo3ΔAD myo5Δ GAL1-MYO3*), Y1899 was transformed with p3147. To generate Y2137 (*bee1ΔAD MYO3 myo5Δ GAL1-MYO3*), Y1899 was transformed with p3145. To generate Y2138 (*BEE1 myo3ΔAD myo5Δ GAL1-MYO3*), Y1900 was transformed with p3147.

Plasmid Construction

Several yeast plasmids were derived from pRS315 (*LEU2*) and pRS316 (*URA3*) (Sikorski and Hieter, 1989). p1754, a pRS315-derived plasmid, encoding Myo3p(SH3-W1157S), was created in four steps. First, we PCR amplified a 450-bp DNA fragment encoding Myo3p-SH3-AD (1118-1271), with primers (5′-CAACCAAAGGATCCGAAATTCGAAGCT-3′) that spanned an internal BamHI site (underlined) and (5′-TCGCGACCAGTCATCATCATCGCCATCGT-3′) that incorporated an NruI site. Second, to create p1686, the NruI site within the cloned product allowed blunt end addition of a linker (5′-TAGGCGGCCGCTA-3′), such that the COOH-terminal codon of Myo3p was followed by a serine codon, a termination condon, and a NotI site. Third, the insert of p1686 provided a template for site specific mutagenesis with an oligonucleotide (5′-CTTTGCTAAAGAGCTCCCACTAGGTTCATC-3′) that changed a tryptophan codon to a serine codon and incorporated an SstI site. Finally, the product was ligated into pVL96 to create p1754. pVL96 is a pRS315-derived plasmid carrying *MYO3* (Wu et al., 1997). Two steps created p2921, a pRS315-derived plasmid encoding Myo3pΔAD. First, a 210-bp DNA fragment coding for Myo3p-SH3 (1118-1188) was amplified by PCR, with primers (5′-CAACCAAAGGATCCGAAATTCGAAGCT-3′) that spanned an internal BamHI and (5′-GCGGCCGCCTAACGCGTCACCGTATTTCTTGTGTCTTTATA-3′) that incorporated an MluI site, followed by termination codon and a NotI site. Second, the product was ligated into pVL96. pBK28 is a p415GAL1(*LEU2*)-derived plasmid (Mumberg et al., 1994) containing *GAL1-MYO3*. To create pBK28, *MYO3* was amplified by PCR, with primers (5′-TCCCCCGGGATGGCTTGCATAAAAAAGGG-3′) and (5′-TGCGGTCGACAAATTAAGGAGGGTTTACTGTGCT-3′) that incorporated SmaI and SalI sites, and ligated into the p415GAL1 polylinker. p3145 was created by ligating a 5.4-kb SalI to NotI fragment of *MYO3* from pVL96 into pRS316. p3147, a pRS316-derived plasmid encoding Myo3pΔAD, was created by substituting the BamHI to NotI fragment of p3145 with the BamHI to NotI fragment from p2921. To create p1704, encoding GST-Myo3p-SH3-AD, we ligated the insert from p1686 in frame with the GST-sequences of a pGEX-3X–derived vector. To create p2924, encoding GST-Myo3p-SH3, we ligated the p2921 DNA fragment encoding Myo3p-SH3 (1118-1188) in frame with GST-sequences of pGEX-3X. A three-step process was used to create p2925, encoding GST-Myo3p-AD. First, we PCR amplified a 270-bp fragment of *MYO3* with primers (5′-GGATCCAAACAAGAAATACGGTGCCTGTTG-3′ and (5′-TCGCGACCAGTCATCATCATCATCGCCATCGT-3′) that incorporated a BamHI and NruI sites. Second, we replaced the NruI site with linker (5′-TAGGCGGCCGCTA-3′) such that the COOH-terminal codon of Myo3p was followed by a serine codon, a termination condon, and a NotI site. Finally, the product was ligated in frame with the GST-sequences of a pGEX-3X derived vector. A two-step process was used to create p1746, encoding Myo3p-GFP. First, we ligated the 450-bp BamHI to NruI fragment containing Myo3p-SH3-AD (1118-1271) in frame with the GFP sequences carried on a pRS316-derived vector, p1444. A NotI site occurs downstream of the GFP sequence in p1444, which enabled ligation of a BamHI to NotI fragment encoding Myo3p-SH3-AD-GFP into pVL96, creating p1746. To create p1753, encoding Myo3p(SH3-W1157S)-GFP, we ligated the 450-bp BamHI to NruI fragment of Myo3p(SH3-W1157S) into p1746. A two-step process was used to create p2967, encoding Myo3pΔAD-GFP. First, the NruI site in p1746 was changed to MluI with a linker (5′-ATAACGCGTTAT-3′), creating p1880. Second, the BamHI to MluI fragment encoding Myo3p-SH3 (1118-1188) was ligated into p1180, creating p2967.

Two-hybrid constructs were based on pEG202 (Gyuris et al., 1993), which encodes the LexA DNA binding domain, and pJG4-5 (Gyuris et al.,

[1]*Abbreviations used in this paper:* AD, acidic domain; DIC, differential interference contrast; GFP, green fluorescent protein; GST, glutathione-*S*-transferase; HA, hemagglutinin; MBP, maltose binding protein; SH3, Src homology domain 3; WH2, WASP homology domain 2.

1993), which encodes the B42 transcriptional activation domain. p1190 contains an EcoRI fragment encoding Myo3p (1054-1271) that was isolated in a two-hybrid screen with the proline-rich FH1 domain of Bni1p as the bait. DNA fragments of various genes were amplified by the polymerase chain reaction (PCR) with primers that incorporated 5′-BamHI and 3′-NotI restriction sites for insertion into the two-hybrid vectors. pEG202 derived plasmids were: p2601, encoding Arc19p; p2605, encoding Arc40p; p3186, encoding Bee1p (1-633); p3174, encoding Bee1p (1-165); p3037, encoding Bee1p (166-633); p3268, encoding Vrp1p (1-817). pJG4-5–derived plasmids were: p1706, encoding Myo3p-SH3-AD (1118-1271) (Anderson et al., 1998); p1475, encoding Myo5p-SH3-AD (1020-1219); p2923, encoding Myo3p-SH3 (1118-1188); p2926, encoding Myo3p-AD (1182-1271); p3023, encoding Myo3p-ADΔW (1182-1270); p3020, encoding Myo3p-AD-32 (1239-1271); p2819, encoding Bee1p-WH2-AD (531-633); p1740, encoding Myo3p-SH3(W1157S)-AD (1118-1271); p1387 encoding Vrp1p (1-200) (Anderson et al., 1998); p1389 encoding Vrp1p (195-817) (Anderson et al., 1998).

To create p3224 carrying a DNA fragment coding for MBP-Vrp1p (1-200), the insert from p1386 was ligated in frame with the MBP-sequences of pMAL-c2 (New England Biolabs). To create p3349 carrying a DNA fragment coding for MBP-Vrp1p (211-437), a 680-bp fragment of *VRP1* was PCR amplified with primers (5′-GGGAATTCTCTAATATTTCTCTTCCTAGTGTG) and (5′-GGGTCGACTCATACTGACGTTGCAACCGAAGGA) that incorporated EcoRI and SalI sites (underlined), and the product was ligated in frame with the MBP-sequences of pMAL-c2. To create p3385 carrying a DNA fragment coding for MBP-Bee1p (213-222), synthetic oligonucleotides (5′-AATTCGCACCGACTACCCCAGCCCCTGCTCTTCCTG-3′) and (5′-TCGACAGGAAGAGCAGGGGCTGGGGTAGTCGGTGCG-3′) that incorporated EcoRI and SalI sticky ends (underlined) and coded for Bee1p (213-222) were ligated in frame with the MBP-sequences of pMAL-c2.

Two Hybrid Analysis

Two hybrid experiments (Phizicky and Fields, 1995) were performed by mating Y1026 (*Mat***a** *ura3-1::URA3lexAop-lacZ leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*), which contained a LexA DNA-binding domain plasmid, and Y860 (*Matα ura3-1::URA3 lexAop-ADE2 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*), which contained a transcriptional-activation domain plasmid. Diploids were grown in liquid culture to mid-log phase and assayed for expression of *lexAop-lacZ* as described (Hagen et al., 1991). Each value represents the average and standard deviation for three independent quantifications.

Coimmunoprecipitation Experiments

Yeast cells were grown to early log phase and extracts were prepared by liquid nitrogen grinding in lysis buffer (0.1% Triton X-100, 50 mM Tris-HCl, 100 mM NaCl, and 10 mM EDTA) with a protease inhibitor cocktail (10 mM Pefabloc, 50 μg/ml leupeptin, 50 μg/ml pepstatin A, 50 μg/ml E64, 50 μg/ml antipain, 50 μg/ml chymostatin, 20 mM benzamidine, and 50 μg/ml aprotinin from Boehringer Mannheim). Immunoprecipitations were carried out with 1.5-ml extracts, containing 20–25 mg/ml total protein, monoclonal antibody HA.11 (Berkeley Antibody Company), and G–Sepharose beads (Pharmacia). For immunoblot analysis, 25 μg of total protein was loaded for detection of proteins in the yeast extract, 10% of the total immunoprecipitated material was loaded for detection of the immunoprecipitated protein and 90% was loaded for detection of the coimmunoprecipitated protein. The resultant proteins were subjected to immunoblot analysis (Peter et al., 1993), using rabbit polyclonal HA antibody (Berkeley Antibody Company), rabbit polyclonal c-myc antibody (Santa Cruz), and rabbit polyclonal antibodies directed against Myo3p. In each experiment, ~20% of the HA-tagged protein was immunoprecipitated. For the coimmunoprecipitation of Myo3p with Arc40p-HA, Bee1p-HA, and Vrp1p-HA, ~1% of the total Myo3p in the extract coimmunoprecipitated in each case. For Myo3p(SH3-W1157S), ~0.1% coimmunoprecipitated with Arc40p-HA. For Myo3pΔAD, ~1% coimmunoprecipitated with Bee1p-HA and Vrp1p-HA. For Vrp1p-Myc, ~5% coimmunoprecipitated with Bee1p-HA.

Purified GST-Myo3p(952-1114) was used to generate rabbit polyclonal antibodies against Myo3p. pGST-Myo3p(952-1114) was created by blunt end ligation of a BsaAI to NcoI fragment pVL96.1 (Wu et al., 1997) into SmaI site of pGEX-4T2 (Pharmacia). The antigen was then affinity purified by coupling 1 mg of purified GST and GST-Myo3p(952-1114) to a NHS-activated HiTrap column. Both columns were then mounted on a BioCad (Perseptive Biosystems). 5 ml of the polyclonal rabbit anti-Myo3p antiserum was applied to the columns, washed with 50 mM sodium phosphate buffer, and eluted using 0.1 M glycine/HCl pH 2.7. Peak fractions containing the anti-Myo3p antibody were collected and neutralized.

GST Pulldown Experiments

Yeast extracts from strains Y1790 and Y1789 were prepared by growing strains to mid-log in 50 ml culture and then grinding cells with glass beads in lysis buffer (0.1% Triton X-100, 50 mM Tris-HCl, 100 mM NaCl, and 10 mM EDTA). *Escherichia coli* strain BL21 (Novagen) was transformed with pGEX-3X (Pharmacia), p1704, p2924, and p2925, and induced to express GST, GST-Myo3p-SH3-AD, GST-Myo3p-SH3, and GST-Myo3p-AD, respectively. GST proteins were purified on glutathione-Sepharose beads, then mixed with yeast extracts (45 min) and prepared for immunoblot analysis (Evangelista et al., 1997) using monoclonal antibody HA.11 (Berkeley Antibody Company), and monoclonal GST antibody (Santa Cruz). For direct protein–protein interaction experiments, BL21 lysates containing MBP-Vrp1p (1-200) (p3224), MBP-Vrp1p (211-437) (p3349), or MBP-Bee1p (213-222) (p3385) were mixed with purified GST proteins purified from BL21 cells expressing pGEX-3X (Pharmacia), GST-Myo3p-SH3-AD (p1704), or GST-Myo3p-SH3(W1157S)-AD (p1741) and prepared for immunoblot analysis (Evangelista et al., 1997) using monoclonal MBP antibody (New England Biolabs) and monoclonal GST antibody (Santa Cruz).

Identification of Myosin-I SH3 Domain Ligands

A random peptide 2-hybrid library (Yang et al., 1995) was screened with p1190, a pEG202 bait plasmid encoding Myo3p-SH3-AD. From approximately 1.5 million transformants, 2 positives were identified. To confirm these interactions, we synthesized oligonucleotides coding for the identified proline-rich peptides, PRP1 and PRP2, and cloned them into pEG202 for further analysis. Baits coding for derivatives of PRP2, in which alanine residues were substituted for particular prolines, were also constructed. pEG202 peptide bait plasmids were p2674, which contains BamHI to NotI fragment encoding peptide PRP1 (QPDHPPPSPP); p2627, which contains BamHI to NotI fragment encoding peptide PRP2 (PPPYQAPHPP); p2675 which contains BamHI to NotI fragment encoding peptide PRP2-1 (PPPYQAPH**A**P); p2676 which contains BamHI to NotI fragment encoding peptide PRP2-2 (P**A**PYQAPHPP); p2718 which contains BamHI to NotI fragment encoding peptide PRP2-3 (PPPYQA**A**HPP); p2730 which contains BamHI to NotI fragment encoding peptide PRP2-4 (PPPYQAPHP**A**); p2880 which contains a BamHI to NotI fragment encoding a peptide PBS2 (NKPLPPLPVA) that was derived from amino acids 92-101 of Pbs2p. p2637, a pJG4-5–derived plasmid encoding the SH3 domain of Sho1p (176-367), was a positive control for peptide PBS2 (Maeda et al., 1995). Pattern match analysis was carried out at the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/saccharomyces/).

Analysis of Myo3p Tail Mutants and Localization of Myo3p-GFP Derivatives

To assess the function of Myo3p mutants, we transformed RH3384 (*myo5Δ*::*TRP1 myo3Δ*::*HIS3 lys2 his3 leu2 trp1 ura3 bar1*) carrying p*MYO5*, a *URA3*-based plasmid (Geli and Riezman, 1996), with pRS315 or pRS315-derived plasmids coding for Myo3p (p1182), Myo3p-ΔAD (p2921), Myo3p(SH3W1157S) (p1754), Myo3p-GFP (p1746), Myo3pΔAD-GFP(p2967), Myo3p(SH3W1157S)-GFP (p1753). These plasmids carry *MYO3* derivatives under control of the endogenous promoter. Transformants were then transferred to medium containing 5-Fluoroorotic (5-FOA) to select for cells that lack p*MYO5* (Boeke et al., 1987). Viable cells were assayed for doubling time and stained with rhodamine-phalloidin (Molecular Probes) to visualize filamentous actin (Adams and Pringle, 1991). In the RH3384 genetic background, *myo3Δ myo5Δ* double mutants are lethal (Geli and Riezman, 1996) and, as expected, we failed to isolate 5-FOA+ colonies from the RH3384 cells transformed with pRS315. p1182, p2921, p1754, p1746, p2967, and p1753 rescued the *myo3Δ myo5Δ* lethality and were associated with a doubling time of 94 ± 4, 92 ± 4, 130 ± 9, 100 ± 4, 92 ± 7, and 166 ± 9 min, respectively; these plasmids resulted in polarization of actin cables and patches towards the growing bud in 96 ± 1, 93 ± 3, 74 ± 8, 96 ± 2, 97 ± 1, and 41 ± 9% of the cells, respectively. For

localization of Myo3p-GFP derivatives, W3031A was transformed with p1746, p2967 and p1753, and observed by fluorescence microscopy with the fluorescein isothiocyanate filter set.

Analysis of bee1ΔAD myo3ΔAD myo5Δ Cells

Growth rate and filamentous actin organization was observed for three strains, Y2136 (*bee1ΔAD myo3ΔAD myo5Δ GAL1-MYO3*), Y2137 (*bee1ΔAD myo5Δ GAL1-MYO3*), and Y2138 (*myo3ΔAD myo5Δ GAL1-MYO3*) after repression of *GAL1-MYO3*. These strains were grown on synthetic medium that selected for the transformed plasmids, but varied in carbon source. For the plate growth assay, cells were propagated on 2% galactose medium, where they express *GAL1-MYO3*, then streaked on 2% glucose medium to score colony formation after *GAL1-MYO3* repression. To visualize the actin cytoskeleton after *GAL1-MYO3* repression, Y2136 cells were grown to mid-log phase in 2% galactose medium, transferred to 2% glucose medium, then stained for filamentous actin with rhodamine-phalloidin (Molecular Probes) as described (Adams and Pringle, 1991). For the *GAL1-MYO3* reinduction experiment, Y2136 cells were first grown to mid-log phase in 2% galactose medium, then shifted to 2% glucose medium for 1 h, washed and shifted to 2% raffinose medium for 27 h, and finally shifted back to 2% galactose medium for 2 h.

Table I. Two-Hybrid Interactions between the Arp2/3 Subunits, Arc19p and Arc40p, and the COOH-terminal Tails of Myo3p, Myo5p, and Bee1p

DNA-binding domain fusion	Activation-domain fusion	LacZ expression (Miller units)
Arc19p	Vector	3.4 ± 0.1
	Myo3p-SH3-AD	152 ± 8
	Myo5p-SH3-AD	133 ± 16
Arc40p	Vector	0.3 ± 0.1
	Myo3p-SH3-AD	458 ± 19
	Myo5p-SH3-AD	347 ± 4
	Bee1p-WH2-AD	18 ± 1
	Myo3p-SH3	1.8 ± 0.2
	Myo3p-AD	151 ± 15
	Myo3p-ADΔW	0.3 ± 0.1
	Myo3p-AD-32	25 ± 1
	Myo3p(SH3-W1157S)-AD	366 ± 15

Assays were done as described (see Materials and Methods); pJG4-5 was the vector control.

Results

COOH-terminal Acidic Domains of Myo3p and Myo5p Interact with the Arp2/3 Complex

The Myo3p and Myo5p COOH-terminal acidic domain (AD) shares homology with the acidic COOH-terminal Arp2/3-binding domain of Bee1p and other WASP-family proteins (Fig. 1). To explore the possibility that myosin-I also associates with the Arp2/3 complex, we constructed two-hybrid baits for each of the yeast subunits (Arp2p, Arp3p, Arc15p, Arc18p, Arc19p, Arc35p, and Arc40p) and performed pair-wise tests for interaction with the myosin-I tails. Initially, we analyzed tails containing both SH3 and acidic domains, Myo3p-SH3-AD and Myo5p-SH3-AD. Two Arp2/3 complex subunits, Arc19p and Arc40p, interacted with the tails of Myo3p and Myo5p (Table I). From a similar analysis of Bee1p COOH-terminal sequences, we detected an interaction with Arc40p specifically (Table I). Because other subunits of the Arp2/3 complex also interact with the Arc19p and Arc40p two-hybrid baits (data not shown), the observed interactions may involve a bridging protein or the entire Arp2/3 complex. Myosin-I and the Arp2/3 complex both localize to cortical actin patches (Goodson et al., 1996; McCollum et al., 1996; Moreau et al., 1996, 1997; Winter et al., 1997, 1999b), suggesting these interactions may occur for full-length proteins in vivo. To test this possibility, hemagglutinin A (HA) epitope-tagged proteins, Arc40p-HA and Arc19p-HA, were immunoprecipitated from yeast extracts and analyzed by Western immunoblotting with antibodies specific for HA and Myo3p. Indeed, we found that Myo3p coimmunoprecipitates specifically with both Arc40p and Arc19p (Fig. 2 A). Approximately 20% of the total Arc40p-HA was immunoprecipitated from the yeast extract and ~1% of the total Myo3p coimmunoprecipitated (Materials and Methods). We conclude that yeast myosin-I proteins associate with the cortical Arp2/3 complex through their COOH-terminal tails.

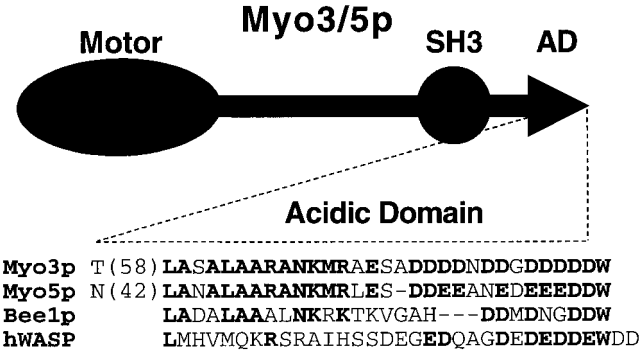

Figure 1. The COOH-terminal acidic domain of yeast myosin-I shares sequence similarity with the COOH-terminal acidic domains of yeast Bee1p and human WASP. The oval represents the head domain of myosin-I, the circle represents the SH3 domain, and the arrow represents the acidic domain.

To identify sequences within the Myo3p tail required for this association, we tested a series of Myo3p tail mutants for Arc40p two-hybrid interactions (Table I). Deletion of the acidic domain leads to a Myo3p tail containing only the SH3 domain, Myo3p-SH3, and abolished interaction with Arc40p. In contrast, a tail containing only the acidic domain, Myo3p-AD, remained competent for the Arc40p interaction. A construct lacking the conserved COOH-terminal tryptophan of the acidic domain, Myo3p-ADΔW, failed to interact with Arc40p, whereas an NH2-truncated acidic domain containing the last 32 residues, Myo3p-AD32, still interacted. Thus, the sequences of Myo3p that share homology with the tail of Bee1p mediate the Arc40p two-hybrid interaction (Fig. 1). Confirmation of these two-hybrid interactions was obtained with glutathione-*S*-transferase (GST)-Myo3p tail fusions purified from *E. coli* and hemagglutinin A (HA) epitope-tagged Arc40p produced in yeast. Myo3p-SH3-AD and Myo3p-AD associated with Arc40p-HA, whereas Myo3p-SH3 did not (Fig. 2 B). Similar experiments confirmed that the Arc19p-HA interaction was also mediated through the acidic domain (Fig. 2 B). Moreover, the acidic domains of Myo3p and Myo5p have been shown to bind directly to purified Arp2/3 complex with an affinity similar to that of the Bee1p acidic domain (Lechler, T., and R. Li, personal communication). These observations suggest the acidic domain

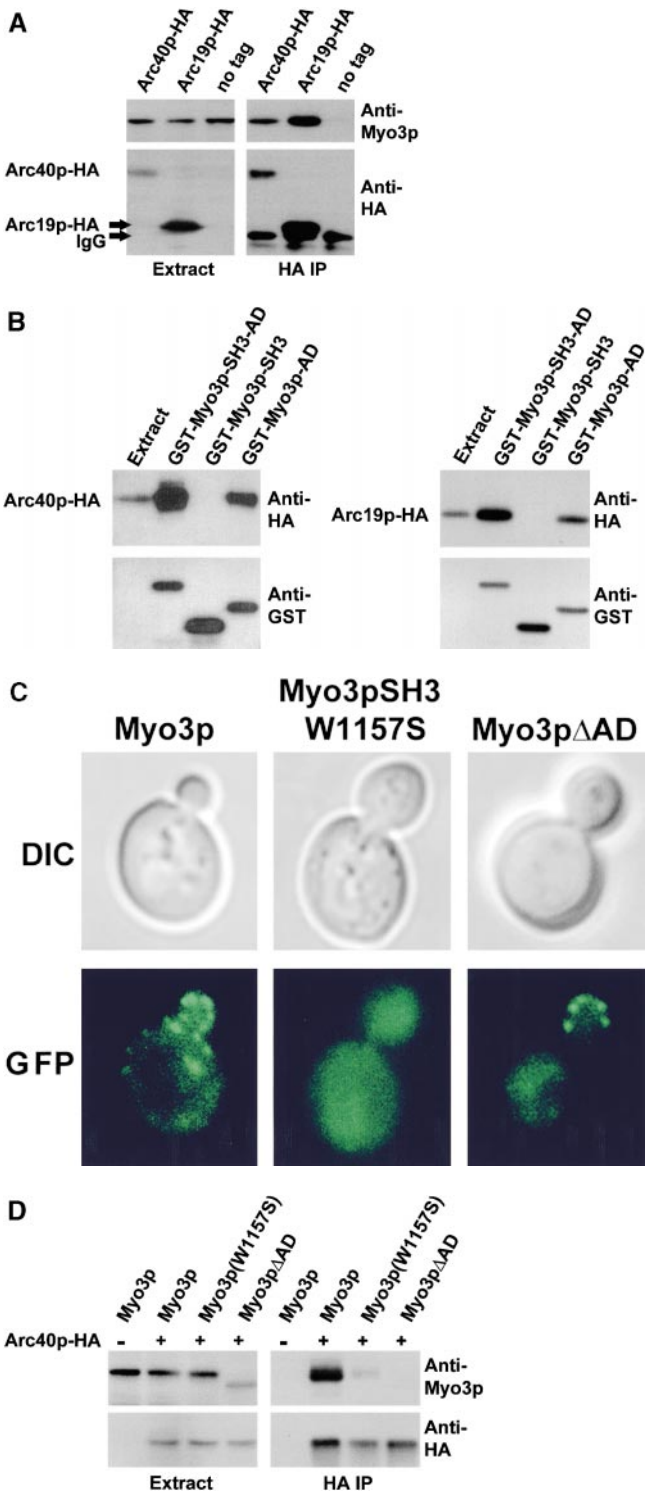

Figure 2. Interaction of Myo3p with subunits of the Arp2/3 complex. (A) Coimmunoprecipitation of Myo3p with Arc40p and Arc19p. Extracts prepared from cells expressing Arc19p-HA, Arc40p-HA, or no tag control, were immunoprecipitated with anti-HA. Immunoprecipitated proteins were detected by immunoblot analysis with antibodies directed against Myo3p (top) or HA (bottom). (B) Interaction of the Myo3p acidic domain with Arc40p and Arc19p. GST-Myo3p-SH3-AD, GST-Myo3p-SH3, or GST-Myo3p-AD bound to glutathione-Sepharose beads was mixed with yeast extracts prepared from cells expressing HA epitope-tagged Arc19p or Arc40p. Bound proteins were detected by immunoblot analysis with antibodies to HA (top) or GST (bottom). (C) Localization of GFP-tagged Myo3p, Myo3p(SH3-W1157S), and Myo3pΔAD. (D) Coimmunoprecipitation of Myo3p with Arc40p. Extracts prepared from cells expressing Arc40p-HA and Myo3p, Myo3p(W1157S), or Myo3pΔAD were immunoprecipitated with anti-HA. Immunoprecipitated proteins were detected by immunoblot analysis with antibodies directed against Myo3p (top) or HA (bottom).

of yeast type I myosins is a protein–protein interaction module that regulates the Arp2/3 complex analogously to the acidic tails of WASP-like proteins (Machesky and Insall, 1998; Egile et al., 1999; Machesky et al., 1999; Rohatgi et al., 1999; Suetsugu et al., 1999; Winter et al., 1999a).

Analysis of Myo3p SH3 Domain and Acidic Domain Mutants

To examine the specific roles of the Myo3p tail domains, we created SH3 and acidic domain mutants. A single amino acid substitution of a conserved tryptophan residue within the SH3 domain resulted in a mutant protein, Myo3p(SH3-W1157S), that was severely mislocalized (Fig. 2 C) and only partially rescued the growth and actin polarity defects of *myo3Δ myo5Δ* cells (Materials and Methods). Similar observations were made for a Myo5p mutant deleted for the SH3 domain (Anderson et al., 1998). In comparison to wild-type Myo3p, a reduced amount of Myo3p (SH3-W1157S) coimmunoprecipitated with Arc40p-HA (Fig. 2 D). This finding was somewhat surprising because the Myo3p(SH3-W1157S) substitution did not abolish the Arc40p two-hybrid interaction (Table I), however, it may be explained by the mislocalization of Myo3p(SH3-W1157S), which could preclude an association with Arc40p in vivo. Thus, the SH3 domain is critical for both function and cortical localization of Myo3p. We also constructed a Myo3p mutant deleted for the acidic domain, Myo3pΔAD. This protein localized to cortical patches (Fig. 2 C) and fully rescued the growth and actin defects of a *myo3Δ myo5Δ* double-mutant but failed to coimmunoprecipitate with Arc40p-HA (Fig. 2 D). Thus, the Myo3p acidic domain is required for the Myo3p-Arc40p association, but is not required for Myo3p localization or function in otherwise wild-type cells.

Myo3p Acidic Domain is Functionally Redundant with the Bee1p Acidic Domain

The COOH-terminal Arp2/3-binding domain of Bee1p is dispensable for its actin assembly function in vivo (Winter et al., 1999a). Therefore, we decided to test if Myo3p and Myo5p formed a complex with Bee1p in which the myosin-I acidic domain was functionally redundant with the Bee1p acidic domain. Indeed, both yeast myosin-I tails showed a two-hybrid interaction with Bee1p (Table II). The Myo3p SH3 domain was necessary and sufficient for the interaction, which occurred through both NH_2- and COOH-terminal fragments of Bee1p (Table II). Coimmunoprecipitation of Myo3p with Bee1p-HA confirmed this was a bona fide interaction in vivo (Fig. 3 A). Further-

more, deletion of the Myo3p acidic domain did not perturb the Myo3p-Bee1p association, whereas the Myo3p (SH3-W1157S) substitution abolished it (Fig. 3 A). Thus, Bee1p associates with the myosin-I SH3 domain.

To test for functional redundancy of the myosin-I and Bee1p Arp2/3-binding domains, we generated *myo5*Δ cells carrying a deletion of the acidic domain of Bee1p, the acidic domain of Myo3p, or both. These cells contained the *MYO3* gene regulated by the *GAL1* promoter, *GAL1-MYO3*, which allowed us to first grow cells on galactose medium, where they expressed *MYO3*, and then shift to glucose medium (a repressing carbon source) to reveal the mutant phenotype. On glucose medium, both the single tail-deletion mutants, *bee1ΔAD* and *myo3ΔAD*, grew normally, whereas the double-mutant, *bee1ΔAD myo3ΔAD*, showed a severe growth defect (Fig. 3 B). After shift of the *bee1ΔAD myo3ΔAD* double-mutant from galactose to glucose, actin patches first depolarized and then appeared to disassemble (Fig. 3 C). Immunoblot analysis of these cells at 0, 5, 10, 15, 20, 25, and 28 h after the shift from galactose to glucose medium revealed the Myo3p level was reduced by ~50% within 5 h after glucose addition, and below detectable levels within 25 h (data not shown). At 0 h, 71 ± 10% of the cells stained for filamentous actin structures and 100% of these cells showed actin cables and patches polarized towards the growing bud; at 15 h, 40 ± 7% stained for filamentous actin structures and 38 ± 3% of these cells were polarized; at 28 h, 2 ± 1% stained for filamentous actin structures, none were polarized, and a fraction of the cells (~30%) appeared to lyse. To determine if the disassembly of filamentous actin structures was reversible, we carried out a *GAL1-MYO3* reinduction experiment. *bee1ΔAD myo3ΔAD* double-mutants were first grown to mid-log phase in galactose medium, then shifted to glucose medium for 1 h, to raffinose medium (a nonrepressing but non-inducing carbon source) for 27 h, and finally back to galactose medium to re-induce expression of *GAL1-MYO3*. Before re-induction of *GAL1-MYO3*, <1% of the cells contained visible filamentous actin; 30 min after galactose addition, 1.7 ± 0.3% cells contained filamentous actin; 2 h after galactose addition, 49.5 ± 3.2% of cells contained filamentous actin and 88.1 ± 4.5% showed polarized cables and patches (Fig. 3 C). Thus, the myosin-I acidic domain plays an essential actin assembly role in cells defective for Bee1p-mediated Arp2/3-activation.

Myo3p and Myo5p SH3 Domains Bind Proline-rich Ligands in Bee1p and Vrp1p

The tails of Myo3p and Myo5p, containing both the SH3 and the acidic domains, also display two-hybrid interactions with Vrp1p (Verprolin) (Anderson et al., 1998), a proline-rich actin-binding protein that shows genetic and two-hybrid interactions with Bee1p (Naqvi et al., 1998). The Vrp1p-Bee1p two-hybrid interaction (Table II) was confirmed in vivo by coimmunoprecipitation of Myc epitope-tagged Vrp1p, Vrp1p-Myc, with Bee1p-HA (Fig. 4 A). The Vrp1p-Bee1p complex appears to be conserved both structurally and functionally with human WIP-WASP (Ramesh et al., 1997; Vaduva et al., 1999). We

Table II. Two-Hybrid Interactions between Bee1p, Vrp1p, and the COOH-terminal tails of Myo3p and Myo5p

DNA-binding domain fusion	Activation-domain fusion	LacZ expression (Miller units)
Bee1p (1-633)	Vector	0.4 ± 0.1
	Myo5p-SH3-AD	398 ± 27
	Myo3p-SH3-AD	715 ± 125
	Myo3p(SH3-W1157S)-AD	0.3 ± 0.1
	Myo3p-SH3	1,765 ± 209
	Myo3p-AD	0.4 ± 0.1
	Vrp1p (1-200)	0.3 ± 0.1
	Vrp1p (195-817)	144 ± 3
Bee1p (1-165)	Vector	0.1 ± 0.1
	Myo3p-SH3-AD	1,201 ± 117
	Vrp1p (1-200)	0.1 ± 0.1
	Vrp1p (195-817)	144 ± 10
Bee1p (166-633)	Vector	0.4 ± 0.1
	Myo3p-SH3-AD	113 ± 12
	Vrp1p (1-200)	0.4 ± 0.1
	Vrp1p (195-817)	0.5 ± 0.1
Vrp1p (1-817)	Vector	0.1 ± 0.1
	Myo5p-SH3-AD	373 ± 9
	Myo3p-SH3-AD	2,252 ± 194
	Myo3p(SH3-W1157S)-AD	0.1 ± 0.1
	Myo3p-SH3	766 ± 94
	Myo3p-AD	0.1 ± 0.1

Assays were done as described (see Materials and Methods); pJG4-5 was the vector control. Full-length Bee1p (1-633), full-length Vrp1p (1-817), and NH_2- and COOH-terminal fragments were analyzed.

observed that the Myo3p SH3 domain was necessary and sufficient for the Myo3p-Vrp1p two-hybrid interactions (Table II). These findings were confirmed through coimmunoprecipitation experiments involving Vrp1p-HA and Myo3p tail mutants (Fig. 4 B). Deletion of the Myo3p acidic domain did not affect association with Vrp1p, whereas the Myo3p(SH3-W1157S) substitution abolished it (Fig. 4 B). Myo5p also forms a cortical complex with Vrp1p (Anderson et al., 1998). Thus, myosin-I proteins associate with both Vrp1p and Bee1p in an SH3-dependent manner.

To identify ligands specific for the myosin-I SH3 domains, we screened a two-hybrid random peptide library for interaction with a bait containing the Myo3p tail. Two proline-rich peptides, PRP1 and PRP2, were identified and confirmed to interact with the tails of both Myo3p and Myo5p (Table III). This interaction was specific because the myosin-I tails failed to interact with a peptide, PBS2, that binds the Sho1p SH3 domain (Table III). Moreover, the Myo3p(SH3-W1157S) substitution abolished the interaction, indicating that it was dependent upon SH3 domain function. Several derivatives of PRP2 were also tested for interaction with the Myo3p tail (Table III). Our findings indicate that PxxxxPxxP (P is proline, x is any amino acid) represents a minimal consensus sequence for both the Myo3p and the Myo5p SH3 ligand. Pattern match analysis of all predicted yeast proteins showed that Bee1p has 4 potential Myo3/5p SH3 ligands and Vrp1p has 17 potential ligands, the most abundant of any yeast protein. Indeed, we found that maltose binding protein (MBP) fusions to Vrp1p (1-200), Vrp1p (211-437), and Bee1p (213-222) bound directly to a GST fusion of Myo3p-SH3-AD but not

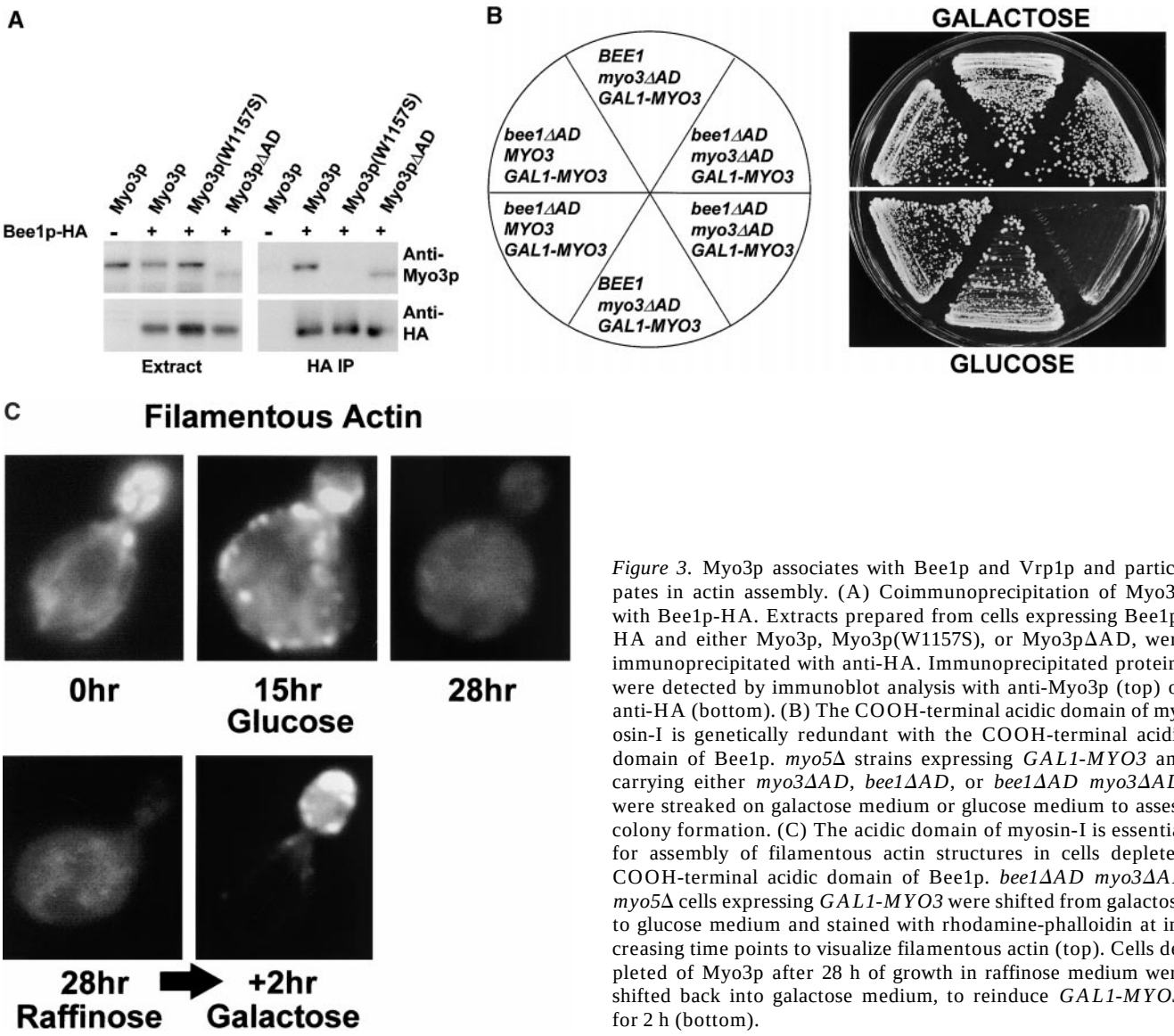

Figure 3. Myo3p associates with Bee1p and Vrp1p and participates in actin assembly. (A) Coimmunoprecipitation of Myo3p with Bee1p-HA. Extracts prepared from cells expressing Bee1p-HA and either Myo3p, Myo3p(W1157S), or Myo3pΔAD, were immunoprecipitated with anti-HA. Immunoprecipitated proteins were detected by immunoblot analysis with anti-Myo3p (top) or anti-HA (bottom). (B) The COOH-terminal acidic domain of myosin-I is genetically redundant with the COOH-terminal acidic domain of Bee1p. *myo5*Δ strains expressing *GAL1-MYO3* and carrying either *myo3ΔAD*, *bee1ΔAD*, or *bee1ΔAD myo3ΔAD*, were streaked on galactose medium or glucose medium to assess colony formation. (C) The acidic domain of myosin-I is essential for assembly of filamentous actin structures in cells depleted COOH-terminal acidic domain of Bee1p. *bee1ΔAD myo3ΔAD myo5*Δ cells expressing *GAL1-MYO3* were shifted from galactose to glucose medium and stained with rhodamine-phalloidin at increasing time points to visualize filamentous actin (top). Cells depleted of Myo3p after 28 h of growth in raffinose medium were shifted back into galactose medium, to reinduce *GAL1-MYO3*, for 2 h (bottom).

to Myo3p-SH3(W1157S)-AD (Fig. 4 C). The proline rich domains of Bee1p and Vrp1p may thus function as a scaffold for assembly of multiple myosin-I molecules. During steady-state ATP hydrolysis, myosin-I, like skeletal muscle myosin-II, is weakly bound to an actin filament (Ostap and Pollard, 1996); therefore, multiple myosin-I molecules must be clustered to support processive motility along actin filaments (Albanesi et al., 1985; Adams and Pollard, 1986; Zot et al., 1992; Ostap and Pollard, 1996).

Discussion

We suggest that Myo3p, Myo5p, Vrp1p, and Bee1p form a complex that controls Arp2/3-mediated actin assembly (Fig. 5). The WH2 (WASP homology 2) domain of WASP-like proteins binds globular actin and is required for efficient actin nucleation in vitro, suggesting it may couple actin monomers to the Arp2/3 complex (Machesky and Insall, 1998; Egile et al., 1999; Machesky et al., 1999; Rohatgi et al., 1999; Suetsugu et al., 1999; Winter et al., 1999a). Bee1p and Vrp1p each contain a WH2 domain (Fig. 5); therefore, the WH2 domain and the acidic Arp2/3-binding domain of Bee1p would be functionally redundant within a Myo3/5p-Vrp1p-Bee1p complex, providing an explanation for the surprising lack of phenotype associated with a Bee1p mutant truncated for both these domains (Winter et al., 1999a). In gel filtration experiments, we find that ~10% of the soluble Arc40p-HA elutes in high molecular mass fractions that also contain Myo3p, Myo5p, and Bee1p-HA (unpublished observations).

Activation of Myo3p and Myo5p requires phosphorylation of the head domain by Ste20p or Cla4p, PAK-like kinases that function as effectors of Cdc42p(GTP), suggesting myosin-I links Cdc42p signaling to Arp2/3-mediated actin assembly (Wu et al., 1996, 1997; Eby et al., 1998). In a variation of this theme, Cdc42p(GTP) binds directly to mammalian N-WASP to control the activity of the Arp2/3 complex (Rohatgi et al., 1999). In yeast, the components of the Myo3/5p-Vrp1p-Bee1p complex and the Arp2/3 complex appear to be particularly important for endocytosis

(Munn et al., 1995; Geli and Riezman, 1996; Geli et al., 1998; Naqvi et al., 1998; Winter et al., 1999b), supporting a role for actin assembly in the formation and movement of endocytotic vesicles (Merrifield et al., 1999).

A carboxyl-terminal acidic domain is present only in myosin-I proteins of *S. cerevisiae*, *Candida albicans*, *Schizosaccharomyces pombe*, and *Aspergillus nidulans*, suggesting a direct interaction with myosin-I and the Arp2/3 complex may be fungal specific. However, in view of both the functional conservation of the Vrp1p-Bee1p-Arp2/3 interactions and the structural conservation of myosin-I SH3 domains, we anticipate that metazoan myosin-I motors are also linked to Arp2/3 complexes through an association with WASP-like adapters. Indeed, the absence of a metazoan acidic domain may reflect the observed functional redundancy in yeast. In further support of a role for myosin-I in actin assembly, we have also detected a Myo3/5p-Bni1p interaction (unpublished observations), which suggests that formins and other adapter proteins may employ myosin-I motors during the assembly of specific actin structures.

Dynamic arrays of actin filaments involved in cell movement, morphogenesis, and other motility processes, must be continually assembled at growing barbed ends and depolymerized at the pointed ends (Drubin and Nelson, 1996; Mitchison and Cramer, 1996; Mullins et al., 1998a,b; Machesky and Insall, 1998, 1999; Suetsugu et al., 1999; Svitkina and Borisy, 1999). Myosin-I moves towards the barbed end of actin filaments and may function to transport WASP-like adapter-Arp2/3 machinery towards sites of active assembly. In addition to their role in nucleation, WASP-like adapters may be involved in the subsequent elongation of filaments (Egile et al., 1999), raising an alternative possibility that myosin-I plays an active role in actin polymerization. Because the barbed ends of filaments tend to orient towards the nucleating site (Zigmond et al., 1998), myosin-I motors could tether polymerizing filaments to assembly machinery and control new monomer addition or filament organization (Mitchison, 1992; Sheetz et al., 1992). Analogous functions have been proposed for

Table III. Two-Hybrid Interactions between Proline-rich Peptides and the COOH-terminal Tails of Myo3p and Myo5p

DNA-binding domain fusion	Activation-domain fusion	LacZ expression (Miller units)
QPDHPPPSPP (PRP1)	Vector	0.2 ± 0.1
	Myo5p-SH3-AD	406 ± 33
	Myo3p-SH3-AD	225 ± 8
	Myo3p(SH3-W1157S)-AD	0.2 ± 0.1
PPPYQAPHPP (PRP2)	Vector	0.2 ± 0.1
	Myo5p-SH3-AD	255 ± 31
	Myo3p-SH3-AD	210 ± 10
	Myo3p(SH3-W1157S)-AD	0.2 ± 0.1
NKPLPPLPVA (PBS2)	Vector	0.3 ± 0.1
	Myo5p-SH3-AD	0.2 ± 0.1
	Myo3p-SH3-AD	0.3 ± 0.1
	Sho1p-SH3	190 ± 28
PPPYQAPH**A**P (PRP2-1)	Vector	0 .3 ± 0.1
	Myo3p-SH3-AD	219 ± 20
P**A**PYQAPHPP (PRP2-2)	Vector	0.2 ± 0.1
	Myo3p-SH3-AD	0.3 ± 0.1
PPPYQA**A**HPP (PRP2-3)	Vector	0.2 ± 0.1
	Myo3p-SH3-AD	0.3 ± 0.1
PPPYQAPHP**A** (PRP2-4)	Vector	0.2 ± 0.1
	Myo3p-SH3-AD	2.8 ± 0.4

Assays were done as described (see Materials and Methods); pJG4-5 was the vector control. For PRP2 derivatives, the proline substitution is indicated as a bold character.

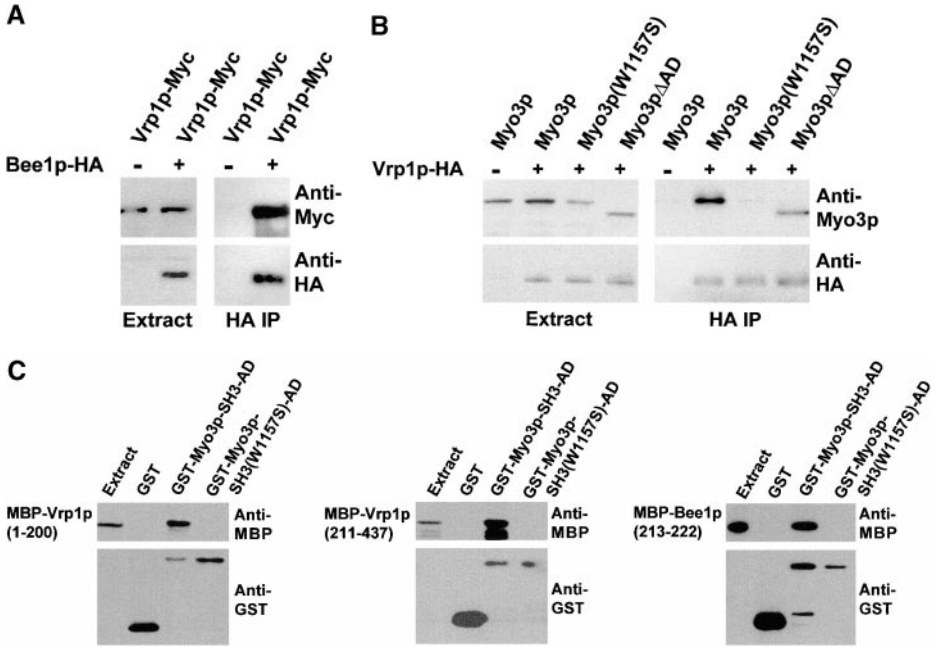

Figure 4. Myosin-I SH3 domain binds proline-rich ligands in Bee1p and Vrp1p. (A) Coimmunoprecipitation of Vrp1p-Myc with Bee1p-HA. Extracts prepared from cells expressing Vrp1p-Myc and Bee1p-HA were immunoprecipitated with anti-HA. Immunoprecipitated proteins were detected by immunoblot analysis with anti-Myc (top) or anti-HA (bottom). (B) Coimmunoprecipitation of Myo3p with Vrp1p-HA. Extracts prepared from cells expressing Vrp1p-HA and either Myo3p, Myo3p(SH3-W1157S), or Myo3pΔAD were immunoprecipitated with anti-HA. Immunoprecipitated proteins were detected by immunoblot analysis with anti-Myo3p (top) or anti-HA (bottom). (C) Direct association of myosin-I with Vrp1p and Bee1p. GST, GST-Myo3p-SH3-AD, or GST-Myo3p-SH3(W1157S)-AD bound to glutathione-Sepharose beads was mixed with *E. coli* extracts containing MBP-Vrp1p (1-200), MBP-Vrp1p (211-437), or MBP-Bee1p (213-222). Bound proteins were detected by immunoblot analysis with antibodies to MBP (top) or GST (bottom).

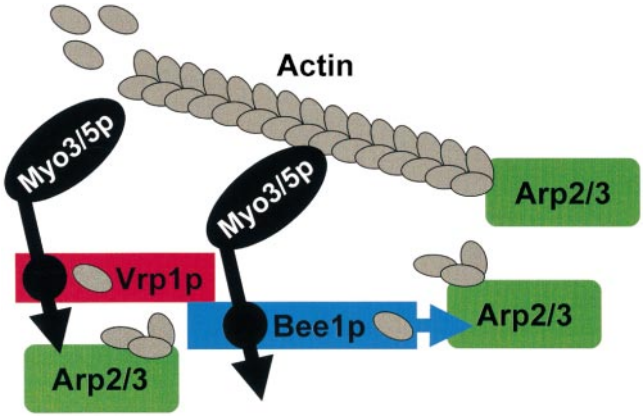

Figure 5. Model for the role of yeast myosin-I in Vrp1p-Bee1p-Arp2/3 mediated actin assembly. The Myo3/5p SH3 domain (circle) binds to Vrp1p and Bee1p. Myo3/5p and Bee1p bind and stimulate the nucleating activity of the Arp2/3 complex through their COOH-terminal acidic domains (arrow). The WH2 domains of Bee1p and Vrp1p bind and couple actin monomers to the Arp2/3 complex for efficient actin nucleation. To represent the WH2 domains, actin monomers are shown bound to Bee1p and Vrp1p. The interaction of the Arp2/3 complex with the regulatory acidic domains of Myo3p, Myo5p, and Bee1p is expected to be transient. Activation of the Arp2/3 complex leads to its incorporation into the actin cytoskeleton, either as a cap on the pointed end of an actin filament or attached to the side of a filament, creating cross-links and branches. The head domain of myosin-I (oval) may organize the newly formed filaments, displace filaments to facilitate barbed-ended insertion, or transport the Vrp1p-Bee1p-Arp2/3 actin assembly complex to the barbed end of a growing actin filament.

plus end–directed microtubule motors in kinetochores (Mitchison, 1992; Wood et al., 1997). By these and possibly other mechanisms, myosin-I may promote the assembly of filamentous actin in a diverse set of motility processes.

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References

Adams, A.E., and J.R. Pringle. 1991. Staining of actin with fluorochrome-conjugated phalloidin. *Methods Enzymol.* 194:729–731.

Adams, R.J., and T.D. Pollard. 1986. Propulsion of organelles isolated from *Acanthamoeba* along actin filaments by myosin-I. *Nature.* 322:754–756.

Albanesi, J.P., H. Fujisaki, J.A. Hammer, III, E.D. Korn, R. Jones, and M.P. Sheetz. 1985. Monomeric *Acanthamoeba* myosins I support movement in vitro. *J. Biol. Chem.* 260:8649–8652.

Anderson, B.L., I. Boldogh, M. Evangelista, C. Boone, L.A. Greene, and L.A. Pon. 1998. The Src homology domain 3 (SH3) of a yeast type I myosin, Myo5p, binds to verprolin and is required for targeting to sites of actin polarization. *J. Cell Biol.* 141:1357–1370.

Baines, I.C., H. Brzeska, and E.D. Korn. 1992. Differential localization of *Acanthamoeba* myosin I isoforms. *J Cell Biol.* 119:1193–1203.

Beckerle, M.C. 1998. Spatial control of actin filament assembly: lessons from *Listeria*. *Cell.* 95:741–748.

Bi, E., and S.H. Zigmond. 1999. Actin polymerization: where the WASP stings. *Curr. Biol.* 9:R160–R163.

Boeke, J.D., J. Trueheart, G. Natsoulis, and G.R. Fink. 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* 154:164–175.

Brzeska, H., J. Szczepanowska, J. Hoey, and E.D. Korn. 1996. The catalytic domain of *Acanthamoeba* myosin I heavy chain kinase. II. Expression of active catalytic domain and sequence homology to p21-activated kinase (PAK). *J. Biol. Chem.* 271:27056–27062.

Drubin, D.G., and W.J. Nelson. 1996. Origins of cell polarity. *Cell.* 84:335–344.

Eby, J.J., S.P. Holly, F. van Drogen, A.V. Grishin, M. Peter, D.G. Drubin, and K.J. Blumer. 1998. Actin cytoskeleton organization regulated by the PAK family of protein kinases. *Curr. Biol.* 8:967–970.

Egile, C., T.P. Loisel, V. Laurent, R. Li, D. Pantaloni, P.J. Sansonetti, and M.-F. Carlier. 1999. Activation of the Cdc42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. *J. Cell Biol.* 146:1319–1332.

Evangelista, M., K. Blundell, M.S. Longtine, C.J. Chow, N. Adames, J.R. Pringle, M. Peter, and C. Boone. 1997. Bni1p, a yeast formin linking Cdc42p and the actin cytoskeleton during polarized morphogenesis. *Science.* 276:118–122.

Frazier, J.A., and C.M. Field. 1997. Actin cytoskeleton: are FH proteins local organizers? *Curr. Biol.* 7:R414–R417.

Geli, M.I., and H. Riezman. 1996. Role of type I myosins in receptor-mediated endocytosis in yeast. *Science.* 272:533–535.

Geli, M.I., A. Wesp, and H. Riezman. 1998. Distinct functions of calmodulin are required for the uptake step of receptor-mediated endocytosis in yeast: the type I myosin Myo5p is one of the calmodulin targets. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:635–647.

Gertler, F.B., K. Niebuhr, M. Reinhard, J. Wehland, P. Soriano. 1996. Mena, a relative of VASP and *Drosophila* Enabled, is implicated in the control of microfilament dynamics. *Cell.* 87:227–239.

Goodson, H.V., and J.A. Spudich. 1995. Identification and molecular characterization of a yeast myosin I. *Cell Motil. Cytoskeleton.* 30:73–84.

Goodson, H.V., B.L. Anderson, H.M. Warrick, L.A. Pon, and J.A. Spudich. 1996. Synthetic lethality screen identifies a novel yeast myosin I gene (*MYO5*): myosin I proteins. *J. Cell Biol.* 133:1277–1291.

Gyuris, J., E. Golemis, H. Chertkov, and R. Brent. 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell.* 75:791–803.

Hagen, D.C., G. McCaffrey, and G.F. Sprague, Jr. 1991. Pheromone response elements are necessary and sufficient for basal and pheromone-induced transcription of the *FUS1* gene of *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 11:2952–2961.

Jung, G., X. Wu, and J.A. Hammer, III. 1996. *Dictyostelium* mutants lacking multiple classic myosin I isoforms reveal combinations of shared and distinct functions. *J. Cell Biol.* 133:305–323.

Lee, S.F., T.T. Egelhoff, A. Mahasneh, and G.P. Cote. 1996. Cloning and characterization of a *Dictyostelium* myosin I heavy chain kinase activated by Cdc42 and Rac. *J. Biol. Chem.* 271:27044–27048.

Longtine, M.S., A. McKenzie, III, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philippsen, and J.R. Pringle. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast.* 14:953–961.

Machesky, L.M., and R.H. Insall. 1998. Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr. Biol.* 8:1347–1356.

Machesky, L.M. 1999. Rocket-based motility: a universal mechanism? *Nature Cell Biol.* 1:E29–E31.

Machesky, L.M., and R.H. Insall. 1999. Signaling to actin dynamics. *J. Cell Biol.* 146:267–272.

Machesky, L.M., R.D. Mullins, H.N. Higgs, D.A. Kaiser, L. Blanchoin, R.C. May, M.E. Hall, and T.D. Pollard. 1999. Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. *Proc. Natl. Acad. Sci. USA.* 96:3739–3744.

Machesky, L.M., and K.L. Gould. 1999. The Arp2/3 complex: a multifunctional actin organizer. *Curr. Opin. Cell Biol.* 11:117–121.

Maeda, T., M. Takekawa, and H. Saito. 1995. Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. *Science.* 269:554–558.

McCollum, D., A. Feoktistova, M. Morphew, M. Balasubramanian, and K.L. Gould. 1996. The *Schizosaccharomyces pombe* actin-related protein, Arp3, is a component of the cortical actin cytoskeleton and interacts with profilin. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:6438–6446.

McGoldrick, C.A., C. Gruver, and G.S. May. 1995. *myoA* of *Aspergillus nidulans* encodes an essential myosin I required for secretion and polarized growth. *J. Cell Biol.* 128:577–587.

Mermall, V., P.L. Post, and M.S. Mooseker. 1998. Unconventional myosins in cell movement, membrane traffic, and signal transduction. *Science.* 279:527–533.

Merrifield, C.J., S.E. Moss, C. Ballestrem, B.A. Imhof, G. Giese, I. Wunderlich, and W. Almers. 1999. Endocytic vesicles move at the tips of actin tails in cultured mast cells. *Nature Cell Biol.* 1:72–74.

Mitchison, T.J. 1992. Compare and contrast actin filaments and microtubules. *Mol. Biol. Cell.* 3:1300–1315.

Mitchison, T.J., and L.P. Cramer. 1996. Actin-based cell motility and cell locomotion. *Cell*. 84:371–379.

Moreau, V., A. Madania, R.P. Martin, and B. Winsor. 1996. The *Saccharomyces cerevisiae* actin-related protein Arp2 is involved in the actin cytoskeleton. *J. Cell Biol*. 134:117–132.

Moreau, V., J.M. Galan, G. Devilliers, R. Haguenauer-Tsapis, and B. Winsor. 1997. The yeast actin-related protein Arp2p is required for the internalization step of endocytosis. *Mol. Biol. Cell*. 8:1361–1375.

Mullins, R.D., W.F. Stafford, and T.D. Pollard. 1997. Structure, subunit topology, and actin-binding activity of the Arp2/3 complex from *Acanthamoeba*. *J. Cell Biol.* 136:331–343.

Mullins, R.D., J.F. Kelleher, J. Xu, and T.D. Pollard. 1998a. Arp2/3 complex from *Acanthamoeba* binds profilin and cross-links actin filaments. *Mol. Biol. Cell*. 9:841–852.

Mullins, R.D., J.A. Heuser, and T.D. Pollard. 1998b. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl. Acad. Sci. USA*. 95: 6181–6186.

Mumberg, D., R. Muller, and M. Funk. 1994. Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res.* 22:5767–5768.

Munn, A.L., B.J. Stevenson, M.I. Geli, and H. Riezman. 1995. *end5, end6*, and *end7*: mutations that cause actin delocalization and block the internalization step of endocytosis in *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* 6:1721–1742.

Naqvi, S.N., R. Zahn, D.A. Mitchell, B.J. Stevenson, and A.L. Munn. 1998. The WASp homologue Las17p functions with the WIP homologue End5p/verprolin and is essential for endocytosis in yeast. *Curr. Biol.* 8:959–962.

Novak, K.D., M.D. Peterson, M.C. Reedy, and M.A. Titus. 1995. *Dictyostelium* myosin I double mutants exhibit conditional defects in pinocytosis. *J. Cell Biol.* 131:1205–1221.

Novak, K.D., and M.A. Titus. 1998. The myosin I SH3 domain and TEDS rule phosphorylation site are required for in vivo function. *Mol. Biol. Cell.* 9:75–88.

Ostap, E.M., and T.D. Pollard. 1996. Biochemical kinetic characterization of the *Acanthamoeba* myosin-I ATPase. *J. Cell Biol*. 132:1053–1060.

Peter, M., A. Gartner, J. Horecka, G. Ammerer, and I. Herskowitz. 1993. *FAR1* links the signal transduction pathway to the cell cycle machinery in yeast. *Cell*. 73:747–760.

Phizicky, E.M., and S. Fields. 1995. Protein-protein interactions: methods for detection and analysis. *Microbiol. Rev*. 59:94–123.

Ramesh, N., I.M. Anton, J.H. Hartwig, and R.S. Geha. 1997. WIP, a protein associated with Wiskott-Aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells. *Proc. Natl. Acad. Sci. USA*. 94:14671–14676.

Ramesh, N., I.M. Anton, N. Martinez-Quiles, and R.S. Geha. 1999. Waltzing with WASP. *Trends Cell Biol*. 9:15–19.

Rohatgi, R., L. Ma, H. Miki, M. Lopez, T. Kirchhausen, T. Takenawa, and M.W. Kirschner. 1999. The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell*. 97:221–231.

Sheetz, M.P., D.B. Wayne, and A.L. Pearlman. 1992. Extension of filopodia by motor-dependent actin assembly. *Cell Motil. Cytoskel.* 22:160–169.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics.* 122:19–27.

Suetsugu, S., H. Miki, and T. Takenawa. 1999. Identification of two human WAVE/SCAR homologues as general actin regulatory molecules which associate with the Arp2/3 complex. *Biochem. Biophys. Res. Commun.* 260:296–302.

Svitkina, T.M., and G.G. Borisy. 1999. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. *J. Cell Biol*. 145:1009–1026.

Symons, M., J.M. Derry, B. Karlak, S. Jiang, V. Lemahieu, F. McCormick, U. Francke, and A. Abo. 1996. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. *Cell*. 84:723–734.

Titus, M.A. 1998. Coming to grips with a multitude of myosins. *Trends Cell Biol.* 8:171–172.

Vaduva, G., N Martinez-Quiles, I.M. Anton, N.C. Martin, R.S. Geha, A.K. Hopper, and N. Ramesh. 1999. The human WASP-interacting protein, WIP, activates the cell polarity pathway in yeast. *J. Biol. Chem*. 274:17103–17108.

Wagner, M.C., B. Barylko, and J.P. Albanesi. 1992. Tissue distribution and subcellular localization of mammalian myosin I. *J. Cell Biol.* 119:163–170.

Wasserman, S. 1998. FH proteins as cytoskeletal organizers. *Trends Cell Biol.* 8:111–115.

Weiner, O.D., G. Servant, M.D. Welch, T.J. Mitchison, J.W. Sedat, and H.R. Bourne. 1999. Spatial control of actin polymerization during neutrophil chemotaxis. *Nature Cell Biol.* 1:75–81.

Welch, M.D., A.H. DePace, S. Verma, A. Iwamatsu, and T.J. Mitchison. 1997. The human Arp2/3 complex is composed of evolutionarily conserved subunits and is localized to cellular regions of dynamic actin filament assembly. *J. Cell Biol.* 138:375–384.

Welch, M.D., J. Rosenblatt, J. Skoble, D.A. Portnoy, and T.J. Mitchison. 1998. Interaction of human Arp2/3 complex and the *Listeria monocytogenes* ActA protein in actin filament nucleation. *Science.* 281:105–108.

Winter, D., A.V. Podtelejnikov, M. Mann, and R. Li. 1997. The complex containing actin-related proteins Arp2 and Arp3 is required for the motility and integrity of yeast actin patches. *Curr. Biol.* 7:519–529.

Winter, D., T. Lechler, and R. Li. 1999a. Activation of the yeast Arp2/3 complex by Bee1p, a WASP-family protein. *Curr. Biol*. 9:501–504.

Winter, D.C., E.Y. Choe, and R. Li. 1999b. Genetic dissection of the budding yeast Arp2/3 complex: a comparison of the in vivo and structural roles of individual subunits. *Proc. Natl. Acad. Sci. USA*. 96:7288–7293.

Wood, K.W., R. Sakowicz, L.S. Goldstein, and D.W. Cleveland. 1997. CENP-E is a plus end-directed kinetochore motor required for metaphase chromosome alignment. *Cell.* 91:357–366.

Wu, C., S.F. Lee, E. Furmaniak-Kazmierczak, G.P. Cote, D.Y. Thomas, and E. Leberer. 1996. Activation of myosin-I by members of the Ste20p protein kinase family. *J. Biol. Chem*. 271:31787–31790.

Wu, C., V. Lytvyn, D.Y. Thomas, and E. Leberer. 1997. The phosphorylation site for Ste20p-like protein kinases is essential for the function of myosin-I in yeast. *J. Biol. Chem*. 272:30623–30626.

Yang, M., Z. Wu, and S. Fields. 1995. Protein-peptide interactions analyzed with the yeast two-hybrid system. *Nucleic Acids Res.* 23:1152–1156.

Zigmond, S.H., M. Joyce, C. Yang, K. Brown, M. Huang, and M. Pring. 1998. Mechanism of Cdc42-induced actin polymerization in neutrophil extracts. *J. Cell Biol*. 142:1001–1012.

Zot, H.G., S.K. Doberstein, and T.D. Pollard. 1992. Myosin-I moves actin filaments on a phospholipid substrate: implications for membrane targeting. *J. Cell Biol*. 116:367–376.