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Review

In Vivo Function of Class I Myosins

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INTRODUCTION

The discovery of *Acanthamoeba castellanii* class I myosins in 1973 was initially greeted with skepticism as the entrenched view was that only conventional myosins existed. Skepticism was replaced by a period of intense biochemical study using column purification techniques and enzymatic assays available at that time. The late 1980s and early 1990s, the era of molecular biology, were characterized by the growing realization that we had previously been only dimly aware of the sheer multitude of myosins existing in the natural world. It became obvious that the forces of natural selection and evolution had brought about the duplication and divergence of an ancestral mechanochemical enzyme so that it could perform a multiplicity of roles.

No fewer than 15 classes of myosins are now known. Their classification is based on sequence similarities between both the motor and tail domains. The amino terminal domains of class I myosins are highly conserved and contain the catalytic ATPase motor activity, whereas the carboxy terminal tail domains are highly divergent (see Fig. 1A). Lacking a coiled coil domain in the tail region like the one found in class II myosins, they do not form dimers and filaments. The tail contains a light-chain binding domain consisting of from one to several IQ motifs adjacent to the motor. Light chain polypeptides are EF hand proteins and include calmodulin. Binding of calmodulin to this region increases motor activity. Following the IQ motifs is a basic amino acid rich region called Tail Homology 1 (TH1), that has been shown to bind phospholipid vesicles in vitro and may play a role in tethering myosin to membranes in vivo. The TH1 domain distinguishes class I myosins from other myosin classes. Following the TH1 domain is the Tail Homology 2 (TH2) or GPA/ GPQ proline rich domain. A second actin-binding site, which is ATP insensitive, is found in this region. The Tail Homology 3 (TH3) region consists of an SH3 domain. The SH3 domain is thought to mediate binding of the protein to the actin cytoskeleton. Based on amino acid sequence, class I myosins can be subdivided into two main groups: amoeboid class I myosins from lower organisms (but that also include rat myr3 and its human homologue myosin IC) and class I myosins from higher organisms.

Several excellent reviews have been written recently about unconventional myosins in general [Wu et al., 2000], and class I myosins specifically [Coluccio, 1997; Barylko et al., 2000]. The aim of this review is to examine in more detail the in vivo function of class I myosins. Recently, rapid advances in our understanding of the in vivo functions of class I myosins in several select model organisms have occurred. This review focuses on the latest findings in these systems. We will discuss the implications of recent biochemical studies of myosin I function on their ability to perform specific tasks in vivo, then explore the role of the various domains, motifs, and regulatory sites found in amoeboid class I myosins. Next, we will review recent findings that suggest that amoeboid class I myosins are organized in a specialized multiprotein complex that plays an important role in regulating the spatial architecture of the actin

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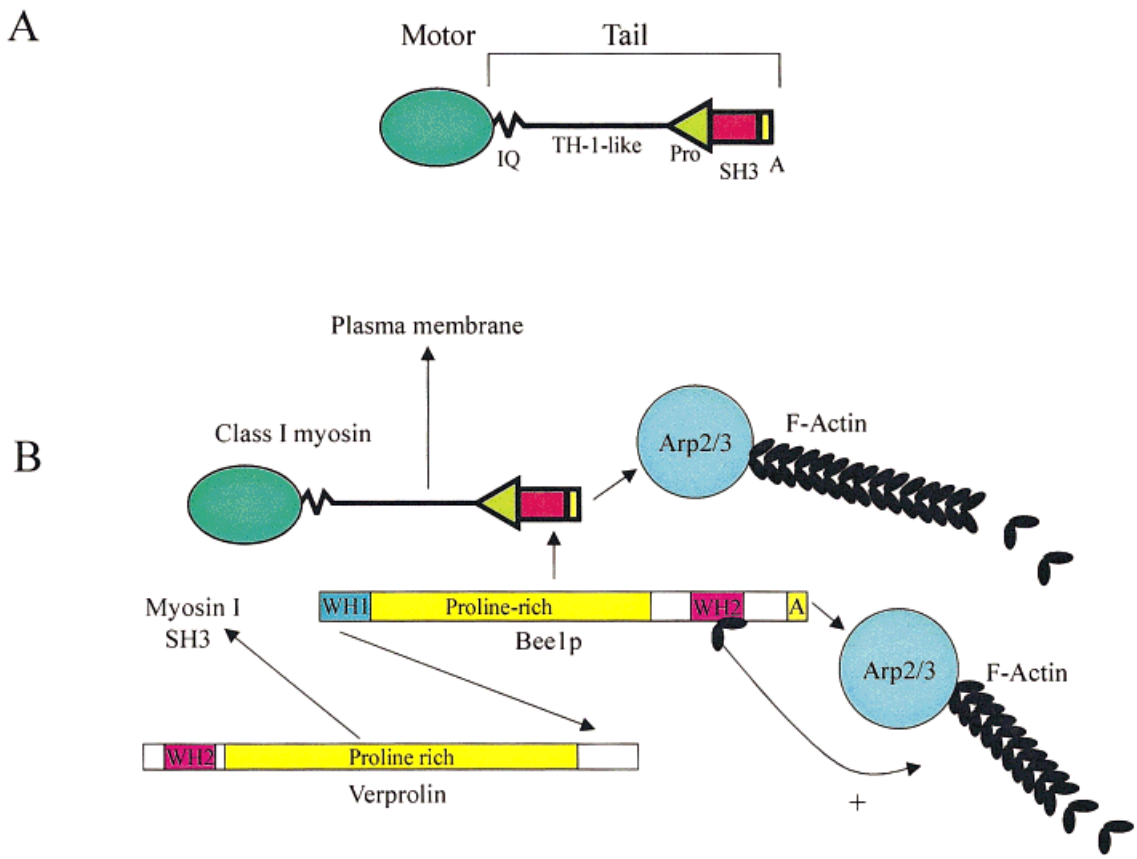


Fig. 1. **A:** Schematic representation of a prototypical class I myosin molecule. The tail is subdivided into the light chain binding domain (IQ motifs), the phospholipid-binding domain (TH1), proline-rich motifs (Pro) and SH3 domain. A cluster of acidic aspartic acid residues (A) is found at the carboxy-terminal end of the tail region of some class I myosins. **B:** Schematic representation of the binding interactions between *S. cerevisiae* myo3p/myo5p and Bee1p, Verprolin, and the Arp2/3 complex of proteins. The myo3p/myo5p SH3 domain binds Bee1p and Verprolin via their proline-rich motifs. The myo3p/myo5p acidic tail region binds the Arp2/3 complex. Bee1p also binds Arp2/3 (via its WH2 domain). The Arp2/3 complex nucleates new actin filament assembly.

cytoskeleton. We will attempt to outline their implications and relevance for the function of mammalian class I myosins.

Despite these important findings, our new knowledge has brought about the realization that we still know little about the in vivo function of the class I myosins. Many important questions remain completely unanswered, and they are raised at the conclusion of this review.

BIOCHEMICAL STUDIES OF MYOSIN I FUNCTION

Kinetic Studies in *Acanthamoeba castellanii* and Their Implications for In Vivo Function

The earliest class I myosins to be studied were isolated from the soil amoeba *A. castellanii* in the 1970s by Pollard and Korn [1973]. This organism expresses three myosin I isoforms, myosin IA, IB, and IC. Under physiological conditions, they are globular monomeric proteins. In the absence of actin, their ATPase motor activity can be activated by the addition of non-physiological levels of Ca^{2+} or K^{+} EDTA. Actin-dependent ATPase activity requires the presence of Mg^{2+} and the phosphorylation of a conserved serine residue in the motor domain [Brzeska et al., 1989]. All three isoforms exhibit a triphasic Mg^{2+} ATPase activity curve as a function of actin concentration [Albanesi et al., 1985; Lynch et al., 1989]. This complex behavior is thought to be a result of cooperative binding of actin to a high-affinity ATP sensitive F-actin binding site in the motor domain and a high-affinity ATP insensitive GPA/GPQ rich F-actin binding site in the tail [Doberstein and Pollard, 1992; Lee et al., 1999; Zot et al., 1999]. Triphasic activation is also seen in *Dictyostelium discoideum* and rat myr3, although surprisingly, the latter lacks a GPA/GPQ-rich region [Lee and Cote, 1993; Stouffler and Bahler, 1998]. Interestingly, *myoA*, the class I myosin

found in the filamentous fungus *Aspergillus nidulans*, displays a simple hyperbolic curve, suggesting that it lacks the high-affinity F-actin binding sites found in the tail [Brzeska et al., 1999].The significance of this finding for in vivo function is unknown.

Biochemical binding studies have also shown that *A. castellanii* myosin I tails bind tightly to negatively charged membranes through the TH1 basic domain [Doberstein and Pollard, 1992; Lee et al., 1999]. This may enable them to tether actin filaments to the membrane. In addition, class I myosins can also cross-link actin cables by simultaneously binding two actin filaments, one to the motor domain on one side and the other to the tail actin-binding region. The hydrodynamic properties of the tail fragment indicate that the SH3 proline-rich domains lie folded across the upstream TH1 region, resulting in a short stubby tail [Lee et al., 1999]. This finding is in agreement with the hypothesis that an intermolecular interaction exists between the SH3 and proline-rich motifs in the tail region, causing them to fold on each other.

Kinetic characterization of *A. castellanii* myosin IA and IB shows them to exhibit the same rapid ATP binding and hydrolysis and slow phosphate release as conventional class II myosin. Most of the cycle is spent in the ADP.Pi-bound state that exhibits low actin-binding affinity. In addition, the in vivo motility of *A. castellanii* myosin I along F-actin fibers is only $\sim 0.2\ \mu ms^{-1}$, about 10–50 fold slower than class II skeletal muscle myosin [Zot et al., 1992]. The hydrodynamic measurements [Lee et al., 1999], three-dimensional structure analysis [Jontes et al., 1998], and kinetic measurements [Ostap and Pollard, 1996a] of *A. castellanii* class I myosins suggest that the reduced actin sliding motility is a result of both the reduced rate of phosphate release and the relatively short, stubby tail that reduces the length of each power stroke. The implication for in vivo function may be that *A. castellanii* myosin I is better suited for short-range cell-surface activities as opposed to long-range activities such as vesicle transport [Ostap and Pollard, 1996a]. In addition, the generation of high local concentrations of myosin I is necessary for continuous movement of actin filaments, and enable it to support such activities as endocytosis and pseudopod extension [Ostap and Pollard, 1996a].

Biochemical Studies of *Dictyostelium discoideum* Class I Myosins

The soil amoeba *D. discoideum* expresses at least six isoforms of class I myosins (MYOA through MYOF). MYO B, C, and D contain tail regions similar to *A. castellanii* myosins I, and are called "long tailed" class I myosins. MYOA and MYOE, lack the SH3 and proline rich domains and are called "short-tailed" myosin I's [Ostap and Pollard, 1996b]. The long tail MYOB and MYOD exhibit triphasic kinetics similar to *A. castellanii* class I myosins [Lee and Cote, 1993], but the short-tailed myosins have not been tested for these functions.

Biochemical Studies of Class I Myosins in Vertebrates

Most of the biochemical studies in vertebrates have been carried out on brush border myosin I (BBMI), which is found exclusively in the lining of the gut. Intestinal epithelial cells contain a brush-like membrane composed of numerous fingerlike protuberances called microvilli. Each microvillus is composed of a core bundle containing actin filaments and radial spoke-like cross-links to the plasma membrane containing BBMI [Matasudaira and Burgess, 1979]. Enzymatic studies of purified BBMI show that it is different from amoeboid class I myosins in four ways: It displays simple hyperbolic, F-actin-dependent, Mg^{2+}ATPase activation, indicating that it lacks an actin-binding site at the tail, and it does not contain the tail SH3 and proline-rich domains found in most amoeboid class I myosins [Collins et al., 1990; Conzelman and Mooseker, 1987]. It does not undergo phosphorylation at the motor domain as it lacks a TEDS-rule phosphorylation site. The TEDS site (named for the conserved amino acid residues T, E, D, and S) occupies a flexible loop in the myosin motor domain of all amoeboid class I myosins. It undergoes serine phosphorylation by p21-activated kinases (PAKS). Phosphorylation activates the motor activity of these myosins. Last, BBMI in-vitro motility is inhibited by Ca^{2+}. This is a result of the dissociation of the light chain, calmodulin, from BBMI [Collins et al., 1990]. Similar biochemical properties have also been seen in rat myr1, the rat homolog of BBMI [Coluccio and Geeves, 1999]. Interestingly, rat myr1 displays a greatly reduced rate of ATP-induced dissociation from actin as compared to other class I myosins, which may indicate that it is primarily designed for the maintenance of cortical tension rather than being involved in movement [Coluccio and Geeves, 1999].

Cryoelectron microscopy of actin filaments decorated with BBMI or smooth muscle myosin (SMM), have revealed substantial differences between them. The BBMI tail undergoes a much larger (~40%) swinging motion than SMM and stays bound to the actin filaments longer during each ATPase cycle [Jontes et al., 1997; Jontes and Milligan, 1997]. Optical tweezer measurements of the work strokes of BBMI and rat myr1 reveal that they move along the actin filament in two discrete phases for each ATPase cycle, the first corresponding to Pi release, and the second one to ADP release [Veigel et al., 1999]. Perhaps the longer swing and lengthier stroke time enable BBMI to carry out a role in vesicle transport in addition to its role as a structural protein.

Regulation of Class I Myosins by PAK

Very early on, it was found that purified *A. castellanii* class I myosin was motor-active only in the presence of a specific kinase-containing column fraction [Maruta and Korn, 1977]. The kinase responsible for this activation was recently cloned and shown to belong to the p21-activated kinase (PAK) family of serine threonine kinases [Brzeska et al., 1996; Daniels and Bokoch, 1999]. Phosphorylation of the *A. castellanii* class I myosins occurs at the highly conserved TEDS rule site positioned at the tip of an actin-binding surface loop in the motor domain. Phosphorylation increases the rate (V_{max}) of actin-dependent phosphate release, thereby increasing actin-dependent ATPase activity roughly 50-fold [Wang et al., 1998]. A similar PAK, called MIHCK (myosin I heavy chain kinase), was also cloned from *D. discoideum* and shown to bind and phosphorylate MYOD in vitro, thereby stimulating its activity [Lee et al., 1996]. MIHCK also binds to the p21 Ras-related proteins Cdc42 and rac. Cdc42 and rac play a key role in controlling cell motility and morphology in many eukaryotic cell types [Hall, 1998]. Therefore, MIHCKs might link cell surface signal transduction pathways that operate through Cdc42 and rac to the machinery that controls the actin cytoskeleton.

The similarities between *A. castellanii* and *D. discoideum* MIHCKs suggest that the signaling pathways controlling class I myosin members have been conserved. Mutation of *A. castellanii* and *A. nidulans* myosin I TEDS sites to either Glu/Asp or Ala to mimic phosphorylation or dephosphorylation, respectively, results in activation or loss of actin activated Mg^{2+}ATPase activity in vitro [Wang et al., 1998] (our unpublished results). This mutation has been a useful tool to analyze the effects of active/inactive class I myosin function in vivo [Wu et al., 1997; Novak and Titus, 1998] (see In Vivo Functions of Class I Myosins). Interestingly, vertebrate class I myosins contain a charged acidic Glu or Asp residue in place of a phosphorylatable serine or threonine at the TEDS sight, thereby mimicking the activated state in a constitutive, unregulated manner. Presumably, motor activity in these myosins is controlled through other undiscovered mechanisms.

Tail of Class I Myosins Is in a Complex With Other Proteins

The discrete subcellular localization of various myosin I's has been attributed to "localization addresses" located within the tail region [Coluccio, 1997]. Binding through SH3 domains was known to link various signal transduction proteins to the cytoskeleton. Acan125 was isolated using a bacterially expressed SH3 fusion protein of *A. castellanii* myosin IC in a cell-extract-binding column to isolate specific SH3-binding proteins from this organism [Xu et al., 1995]. Acan125 was determined to bind to the myosin IC SH3 domain through a proline rich (PXXP) region at its carboxy-terminus [Xu et al., 1997]. Acan125 also contains a carboxy-terminal leucine-rich repeat (LRR) region conserved in the WASP family of proteins that bind and activate the Arp2/3 complex [Svitkina and Borisy, 1999]. Members of the WASP family of proteins serve as adaptors or scaffolds for the binding of actin to Arp 2/3 and capZ. WASP family proteins bind actin monomers via a conserved WH2 (WASP Homology 2) domain, and Arp 2/3 via an acidic carboxy-terminal sequence. Arp2/3 and capZ serve as nucleation points for actin fiber polymerization.

Recently these findings have been strengthened and extended in studies carried out in *Saccharomyces cerevisiae* and *D. discoideum*, shedding new light on the in vivo function of the amoeboid class I myosins. The emerging model is that class I myosins form a complex with WASP-related proteins to control Arp2/3-mediated actin assembly (Fig. 1B). Myosin I proteins have been shown to bind through their SH3 domains to the multiple proline-rich motifs found in WASP-related proteins in both *S. cerevisiae* (Bee1p, Verprolin) and *D. discoideum* (p116) [Evangelista et al., 2000; Jung et al., 1999; Lechler et al., 2000]. In addition to binding Bee1p through their SH3 domain, *S. cerevisiae* myo3p and myo5p bind directly to Arp2/3 by an acidic region in the tail region. This is surprising since both myo3p and myo5p lack the WH2 motif necessary for Arp2/3 activation. Apparently, there is redundancy of binding in the complex: Arp2/3 can bind the complex either through myosin I or Bee1p [Lechler et al., 2000]. The significance of this redundancy is unknown. Importantly, *S. cerevisiae* class I myosins do not carry out a simple structural role in the complex: myo5p lacking motor activity cannot support actin polymerization in a reconstitution assay [Lechler et al., 2000].

How class I myosins participate in the complex is unclear. One possibility is that they are involved in movement of the complex towards sites of actin assembly. Another, the "ratchet" model, proposes that class I myosins link the complex to the membrane by their tail phospolipid-binding TH1 domain, though there is no biochemical evidence that either class I myosin of *S. cerevisiae* binds phospholipids. Movement of myosin along the elongating actin fiber below it would then generate a membrane protrusion, allowing the addition of more G-actin to the barbed end. Precisely how class I myosins carry out this relatively long-range processive movement is a mystery since biochemical measurements have shown they spend most of their time dissociated from actin filaments. Perhaps the multiple myosin I binding sites (up to 20) found in Bee1p or Verprolin simul-

taneously tether a large number of class I myosins, enabling a more processive activity.

IN VIVO FUNCTIONS OF CLASS I MYOSINS

Dictyostelium discoideum Class I Myosins

D. discoideum is well suited for the study of the in vivo function of class I myosins because one can manipulate its genes (i.e., gene deletion or replacement) and quantify the phenotypic consequences using a variety of cellular and motility assays. The organism's drawbacks stem from the fact that it expresses seven class I myosins (MYOA-F, MYOK). This may be an evolutionary consequence of its complex development life cycle and its highly motile nature. An additional complication in interpreting phenotypes resulting from class I myosin knockouts in *D. discoideum* is that they change dramatically depending on whether the cells are grown on solid plates, their more "natural" state, or in liquid culture.

None of the *D. discoideum* myosin I genes are essential for growth. Single gene deletion studies of *D. discoideum* class I myosins indicate there is considerable overlap in function between them although they do not all perform exactly the same functions. For example, deletions of *myoA, B, C* or overexpression of MYOB all cause reduced motility (probably as a result of the reduction in the speed of pseudopod extension) and pinocytosis [Titus et al., 1993; Wessels et al., 1991; Jung et al., 1996; Novak et al., 1995; Jung and Hammer, 1990; Novak and Titus, 1997] (Fig. 2). Quantitative measurement of these functions show that each myosin I isoform makes a different relative contribution to a particular function. Localization studies also indicate considerable overlap in the subcellular distribution of *D. discoideum* class I myosins [Jung et al., 1993; Fukui et al., 1989; Morita et al., 1996; Titus et al., 1995; Schwarz et al., 2000]. For example, MYOB, C, and D all localize to pseudopods at the leading edge of amoebae. None of the *D. discoideum* class I myosins carry out a unique task that does not overlap with that of others in the class. This may simply be a result of our inability to devise specific enough assays to measure the resulting defects and suggests that there may be additional, more subtle differences that will become apparent at higher levels of experimental resolution.

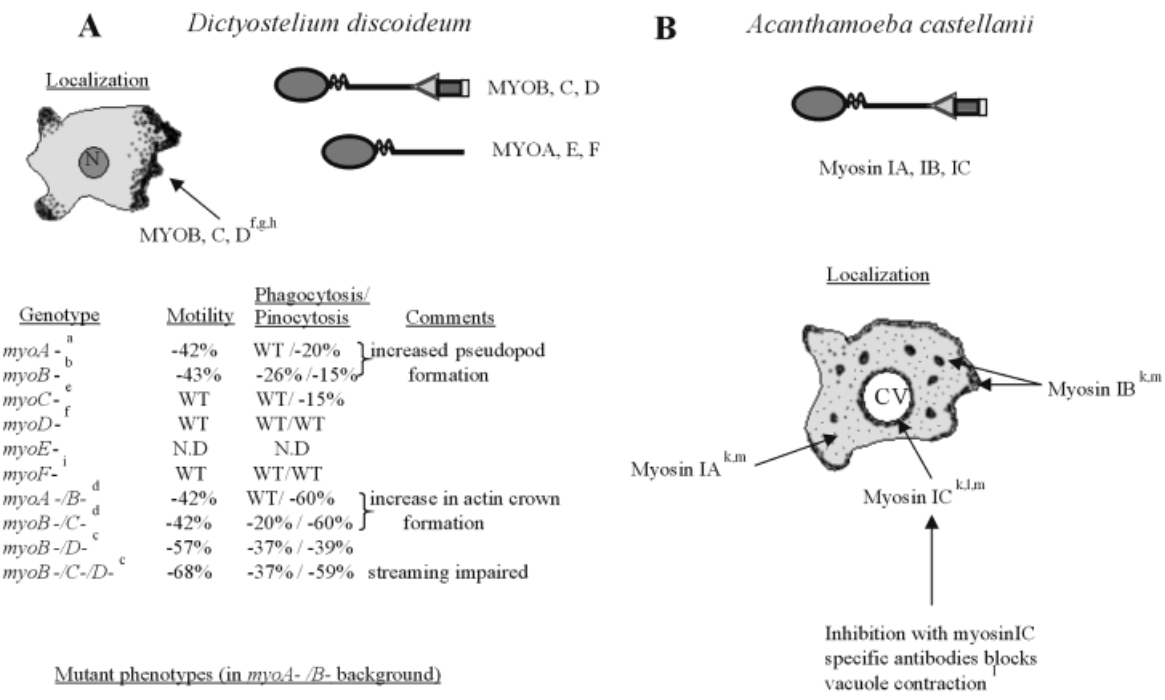
The difficulty of eliciting pronounced morphological defects through single myosin I deletions has led to the construction of strains deleted for multiple myosins (Fig. 2A). For example, a *myoA-/B-* strain shows a synergistic decrease in macropinocytosis (non-receptor-mediated fluid phase uptake, that is carried out by pseudopod extension) and growth rate and a large increase in the formation of actin-rich crowns [Novak et al., 1995]. This mutant was subsequently used to perform mutational studies of MYOB. Mutant versions of MYOB lacking the tail SH3 domain or containing an unphosphorylatable TEDS rule site were unable to rescue the *myoA-/B-* phenotype, but did localize correctly [Novak and Titus, 1998]. This indicates that both the increased motor activity of phosphorylated MYOB and the presence of the SH3 domain are essential for function but not for localization.

The most commonly described model for class I myosin function in vivo in *D. discoideum* is that they provide the motive power to retract actin-rich pseudopods. The function of pseudopods is to pinocytose extracellular fluids prior to their uptake by vesicle transport systems. This model explains the decreased rates of phagocytosis and pinocytosis in myosin I knockout strains and the increased number of pseudopods (as there are no class I myosin molecules to retract them). It also explains the similar defects seen in MYOB overexpressing cells: high levels of MYOB cause retraction to be so strong that the opposing process of pseudopod extension is delayed.

Since motility in these cells is carried out by pseudopod extension and retraction, this too, is affected. The pseudopod retraction model does not directly explain the need for multiple myosin I isoforms. An alternate model is that *D. discoideum* class I myosins have a more general role in optimizing the levels of cortical tension [Dai et al., 1999]. Consistent with this it has been shown that although *D. discoideum* cells lacking only one myosin I have normal levels of cortical tension, double mutants have significantly reduced (50%) cortical tension, and those that overexpress MYOB or MYOC exhibit increased cortical tension [Dai et al., 1999]. Cortical tension could be maintained by myosin I molecules crosslinking and pulling on actin cables. This model does not explain the increase in pseudopod formation in cells lacking a single class I myosin.

A. castellanii Class I Myosins

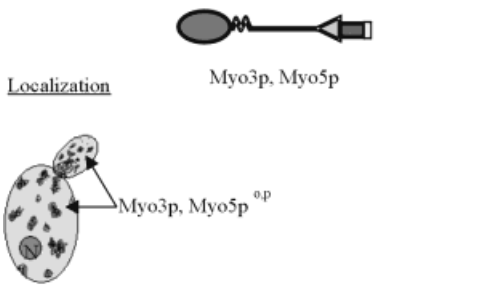
The inability to conduct molecular and genetic manipulations in this organism has limited its utility in the study of in vivo myosin I function. Immunostaining with isoform-specific antibodies has shown that myosins IA, IB, and IC have both distinct and overlapping localization patterns (Fig. 2B) [Baines et al., 1992]. Most striking is the unique localization of myosin IC to the contractile vacuole. This organelle collects and expels excess water when the amoeba is kept under hypotonic conditions. In perhaps the most convincing demonstration of class I myosin in vivo function, amoebae were scrape loaded with myosin IC specific antibodies and shown to lyse under hypotonic stress [Doberstein et al., 1993]. Microscopic analysis revealed that vacuole contractions were



Genotype	Motility	Phagocytosis/ Pinocytosis	Comments
myoA - [a]	-42%	WT /-20%	increased pseudopod formation
myoB - [b]	-43%	-26% /-15%	
myoC - [e]	WT	WT/ -15%	
myoD - [f]	WT	WT/WT	
myoE - [i]	N.D	N.D	
myoF - [i]	WT	WT/WT	
myoA -/B- [d]	-42%	WT/ -60%	increase in actin crown formation
myoB -/C- [d]	-42%	-20% / -60%	
myoB -/D- [c]	-57%	-37% / -39%	
myoB -/C-/D- [c]	-68%	-37% / -59%	streaming impaired

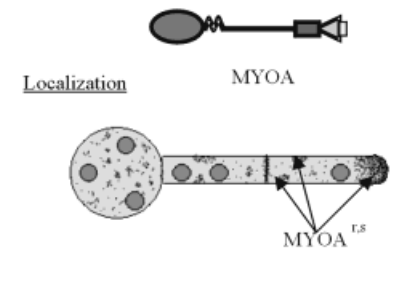
Mutant phenotypes (in *myoA- /B-* background)

Genotype	+/- Complements	Correct localization
myoB S332A [j]	-	+
myoB ΔSH3 [j]	-	+



Genotype	Growth	Endocytosis	Actin patch distribution
myo3 - [n,o]	WT	WT	WT
myo5 -	WT	WT	WT
myo3-/5-	↓↓↓	↓↓	depolarized

Genotype	+/- Complements *myo3-/5-* strain	+/- Correct localization
myo3 S357D [q]	+	+
myo3 S357A [q]	-	ND
myo5 ΔSH3 [p]	+/-	-
myo5 ΔSH3Pro [p]	+/-	-
myo5 ΔPro [p]	+	+
myo5 ΔA [p]	+	+
SH3 alone [p]	-	-



Genotype	Phenotype
myoA null	Nonviable

Genotype	+/- Complements conditional null	+/- Correct localization	Comments
*myoA*S371A [t]	+	+	
*myoA*S371E [s,u]	+	+	increased endocytosis
myoA ΔSH3 [s,u]	+	+	
myoA ΔPro [s,u]	-	-	
myoA ΔA [s,u]	+	+	
myoA ΔIQ [s,t]	-	-	
myoA ΔTH1	-	-	

Figure 2.

specifically inhibited by the antibodies leading to deregulated uptake of water into the cells and subsequent lysis. Interestingly, class I myosins in the amoeboid *D. discoideum* do not co-localize to the contractible vacuole.

The localization of phosphorylated (motor active) and dephosphorylated (motor inactive) myosin I isoforms was determined by Baines et al. [1995] using phospho-specific antibodies. This study showed that the myosin IC associated with the contractile membrane was mostly phosphorylated. Surprisingly, most of the myosin IB and IC associated with the plasma membrane was dephosphorylated and presumably inactive. In contrast, cortex-associated and cytoplasmic myosin I (largely myosin IA) was mostly phosphorylated, especially around phagocytic cups. Taken together, the localization studies of myosin IA and IB indicate a role in maintaining cortical tension (myosin IA) and phagocytosis (myosin IB), although inferring function solely on the basis of localization is risky.

Saccharomyces cerevisiae Class I Myosins

The ability to carry out refined genetic screens and manipulations and the availability of a sequenced genome and a variety of cytoskeleton-related mutants have rapidly pushed *S. cerevisiae* to the forefront of myosin I research. In *S. cerevisiae* there are two highly related class I myosin genes, *myo3* and *myo5*. Deletion of either one results in no observable phenotypic consequences. Deletion of both is either lethal [Geli and Riezman, 1996] or severely debilitating [Goodson et al., 1996], probably depending on the strains genetic background (Fig. 2C). This indicates that myo3p and myo5p probably perform overlapping functions.

S. cerevisiae myo3-/5- double mutant cells exhibit defects in general growth, endocytosis, secretion, actin patch distribution, and changes in cell shape and the cell wall. The mutational analysis of myo3p and myo5p has provided us with several important insights (Fig. 2C). Deletion of the myo5p SH3 domain results in its mislocalization and an inability to complement the *myo3-/5-* phenotype. The tail proline-rich or acidic domains are not essential for in vivo function [Anderson et al., 1998]. Motor activity is absolutely required for myo3p function since a motor inactive S357A TEDS-rule site myo3p mutant is unable to complement the *myo3-/5-* phenotype [Lechler et al., 2000; Geli and Riezman, 1996; Wu et al., 1997]. The motor-activating S357D mutation, containing a charged acidic residue to mimic phosphorylation, can complement the *myo3-/5-* phenotype and support actin assembly in a Cdc42-*1* mutant strain [Lechler et al., 2000]. Cdc42p functions upstream of the PAK family members Cla4p and Ste20p to promote actin polymerization. Surprisingly, introduction of the S357D activating mutation to a strain lacking Cla4p and Ste20p does not rescue the mutant phenotype, suggesting these kinases have other targets that are required for *S. cerevisiae* viability. Although the observed phenotypes of the *myo3-/5-* and mutant strains provide us with some clues as to class I myosin function, it is difficult to pinpoint the primary, as opposed to secondary defects caused by the deletions or mutations. For example, is the slowing of endocytosis caused by defects in the organization and polarity of the actin cytoskeleton, or is it caused directly by the loss of myosin I involvement in the process of endocytosis? If the function of myo3p and myo5p is to participate in the polarized organization of the actin patches and cables, then the observed defects in secretion, endocytosis, and cell shape are secondary, resulting from the loss of actin organization.

Strengthening the argument that *S. cerevisiae* class I myosins are directly involved in actin organization is the finding that both myo3p and myo5p co-localize to actin patches and cables and disperse when actin is artificially depolymerized. Also, both myo3p and myo5p have been shown to form complexes with a variety of actin-binding and organizing proteins (see Biochemical Studies of Myosin I Function). Geli and Riezman [1996] argue that class I myosins are directly involved in endocytosis since defects in endocytosis show up very rapidly ($<$15 min) in a heat-sensitive *myo3-/5- myo5-1* strain at restrictive temperatures (37°C). Unfortunately, at 37°C, the actin cytoskeleton is also rapidly depolarized [Anderson et al., 1998], complicating the interpretation of this experiment.

In our view, the in vivo role of class I myosins will only be accurately defined with the aid of myosin mutant strains. Especially informative will be "separation of function" mutants that have selectively lost their ability to interact with distinct binding partners. This more so-

Fig. 2. Summary of the in vivo functions of amoeboid (**A, B**) and fungal (**C, D**) class I myosins. The class I myosin isoform(s) expressed in each organism and their domain organization are described at the top portion of A–D. (*Oval* = motor domain, *zigzag line* = IQ motifs, *straight line* = TH1 domain, *triangle* = GPA/GPQ proline rich motif, *rectangle* = SH3 domain, *small rectangle* = acidic region.) Cellular localization is given in a schematic representation (Abbreviations are: N, nucleus; CV, contractile vacuole), and relevant citations are given in parenthesis. The phenotypic consequences of class I myosin gene knockouts or mutations are summarized in the tables in the lower portion of each panel. References given in the figure are noted by superscript numbers: [[a]Titus et al., 1993; [b]Wessels et al., 1991; [c]Jung and Hammer, 1996; [d]Novak et al., 1995; [e]Jung and Hammer, 1990; [f]Jung et al., 1993; [g]Fukui et al., 1989; [h]Morita et al., 1996; [i]Schwarz et al., 2000; [j]Novak and Titus, 1998; [k]Baines et al., 1992; [l]Doberstein et al., 1993; [m]Baines et al., 1995; [n]Geli and Riezman, 1996; [o]Goodson et al., 1996; [p]Anderson et al., 1998; [q]Wu et al., 1997; [r]McGoldrick et al., 1995; [s]Yamashita et al., 2000; [t]Yamashita and May, 1998; [u]Osherov et al., 1998].

phisticated approach may well lead us to the conclusion that class I myosins are multifunctional proteins, with diverse binding partners, providing for spatial and temporal regulation of myosin I functions.

Aspergillus nidulans Myosin I

The filamentous fungus *A. nidulans* contains a single class I myosin, *myoA*. MyoA is an essential gene. Both a conditional null mutant and a disrupted heterokaryon undergo cell swelling and nuclear division but fail to initiate hyphal growth. Protein secretion is also reduced [McGoldrick et al., 1995]. Because *A. nidulans* is a genetically amenable organism containing a single essential class I myosin, it is a good system for studying the in vivo function of this class of proteins.

MYOA localizes in actin-associated cortical patches found throughout the fungus, being especially concentrated at the hyphal tip and at sites of septum formation [McGoldrick et al., 1995; Yamashita et al., 2000]. Mutational studies of either the motor or tail domain of MYOA have yielded some surprising results [Yamashita and May, 1998; Osherov et al., 1998].

Mutation of the TEDS rule phosphorylation site from serine to glutamic acid, which mimics phosphorylation, leads to constitutive actin-dependent Mg^{2+}ATPase activity in vitro [Brzeska et al., 1999]. A mutant strain of *A. nidulans* expressing this mutant protein (S371E) shows an accumulation of membranes at the hyphal tip and a sharp increase in the rate of endocytosis. Interestingly a sharp increase in the rate of endocytosis for the corresponding S357D mutant in *S. cerevisiae* has not been reported. The increased rates of endocytosis in the S371E mutant in *A. nidulans* indicate a possible connection between phosphorylation of MYOA by PAK family kinases and involvement in endocytosis. In contrast, mutation of serine 371 to alanine, mimicking the dephosphorylated state of MYOA, resulted in greatly reduced actin-dependent Mg^{2+}ATPase activity (10% of phosphorylated wild type, our unpublished results) but had no effect on endocytosis [Yamashita and May, 1998]. Although growth rates were slightly reduced in liquid submerged culture, the strain was morphologically and structurally normal. Why is this mutant almost normal despite retaining only a fraction of its motor activity? One possibility is that basal motor activity is sufficient to support growth of the fungus. Alternatively, perhaps the motor function of MYOA is not essential for viability; instead, MYOA functions as an essential structural protein required for polarized growth and secretion. Strengthening this argument, we have recently shown that other MYOA "motor-dead" mutations can also support these activities (our unpublished observations). How can the normal endocytosis rates seen in the S371A mutant be explained? One possibility is that endocytosis is a nonessential activity of MYOA. In the absence of MYOA, other proteins, possibly motor proteins, substitute for it.

It is important to note that TEDS rule site motor inactivating mutants of class I myosins in both *D. discoideum* and *S. cerevisiae* are non-functional in vivo, in contrast to our result. Equally surprising, a deletion analysis of the tail subdomains revealed that the SH3 domain of MYOA is not essential for function. MYOAΔSH3was correctly localized in vivo, and the mutant strain was phenotypically normal [Yamashita et al., 2000; Osherov et al., 1998]. On the other hand, deletion of the proline-rich domain resulted in a mutant MYOA that retained only limited function [Osherov et al., 1998]. This mutant grew very poorly and displayed abnormal hyphal branching, suggestive of a failure to initiate and maintain polar hyphal growth. In contrast, in S. cerevisiae, the myo5p SH3 domain is essential for function and localization, and the proline-rich domain is not [Anderson et al., 1998]. In *D. discoideum*, the MYOB SH3 domain is essential for function but not for localization [Novak and Titus, 1998]. How can these differences be explained? Perhaps in *A. nidulans*, MYOA, instead of binding to a proline-rich Bee1p-like docking protein (as in *S. cerevisiae*), binds an SH3 containing protein through its proline-rich motif. Identification of proteins that interact with the MYOA proline-rich motif will resolve this open question. The nonessential MYOA SH3 domain might serve to mask the adjacent proline-rich motif, adding an additional layer of control to the system. It seems that in *A. nidulans*, *myoA*, the single class I myosin gene, is involved in a variety of activities including nonessential functions, such as increasing the rate of endocytosis and essential structural functions in maintaining cell polarity.

Vertebrate Class I Myosins

Most of the studies that have been carried out on the in vivo function of vertebrate class I myosins (BBMI, MMIα, MMIβ, myr1, myr3,) have attempted to imply specific functions based on subcellular localization [Coluccio, 1997]. An especially elegant study identified the localization of different classes of myosins (myosin Iβ, myosin V, myosin VI, myosin VIIa) in the inner ear sensory epithelia [Hasson et al., 1997]. These myosins were found to have both unique and overlapping distributions. For example, myosin Iβ localized primarily to the tips of stereocilia. These are actin-rich protrusions found at the top of hair cells. By virtue of its localization at these tips, myosin Iβ is implicated as the hair cells' "adaptation motor," ensuring that mechanically gated transduction channels are optimally poised to detect tiny deflections induced by sound waves. This study underscores the incredibly complex problem of understanding

how cells differentially use the dozens of myosin isozymes present in the genome.

Several interesting new studies have begun to address the problem of vertebrate myosin I function in vivo by directly perturbing the function of specific myosins and analyzing the resulting outcome. In a notable example, specific destruction of MMIα at the tip of neural growth cones utilizing a technique called CALI (chromophore assisted laser inactivation) resulted in expansion of lammelopodia concentrated at the tip. This finding indicates that MMIα plays a role in maintaining lammelopodial tension [Wang et al., 1996].

Over-expression of the tail region of myr1 in NRK cells revealed that it localized to the plasma membrane like the full-length protein. This finding suggests that the tail contains sufficient information to localize correctly [Ruppert et al., 1995]. In contrast, the BBMI tail region did not localize correctly when overexpressed in a non-polarized hepatoma cell line [Durrbach et al., 1996]. In fact, its mislocalization to diffuse patches in the cytosol was accompanied by defects in endocytosis and structural changes in the endocytic compartments. Similarly, overexpression of truncated BBMI in polarized epithelial cells resulted in perturbation of trafficking of basolaterally internalized molecules to the apical surface [Durrbach et al., 2000]. Whether these dominant effects are a direct result of competition with endogenous full-length myosin I for specific binding sites or a secondary defect remains unclear.

Transformation of HeLa cells with a dominant active form of CdC42 caused a recruitment of myr3 to intracellular junctions, suggesting a link between small G-proteins that regulate the actin cytoskeleton and type I myosins in higher organisms. Interestingly, recruitment of myr3 depended on a functional motor domain and a short region in the tail, but was independent of the SH3/proline rich or TH1 domains [Stoffler et al., 1998].

Conclusions

What generalizations can be made regarding the in vivo functions of class I myosins? The amoeboid class I myosins in *A. castellanii* and *D. discoideum* appear to play a role in actin-dependent movement and changes in shape of subcellular structures such as vacuoles, pseudopods, and endosomes. In fungi (*S. cerevisaie* and *A. nidulans*), the main role may be in supporting polarized growth and endocytosis. Fungi have a protective chitin-rich cell wall surrounding the plasma membrane and do not form pseudopods. Instead, polarized growth may be viewed as a process of directional pseudopod-like extension behind an armoured outer coat. Emerging from these generalizations, class I myosins probably carry out multiple tasks in any given organism. This is evident from the different functions and subcellular distributionof the various class I myosins in *A. castellanii* and *D. discoideum. A. nidulans* MYOA, the only class I myosin expressed in this fungus, carries out several functions, both essential (polarized hyphal growth) and nonessential (increasing the rate of endocytosis). To carry out multiple functions, class I myosins probably form different protein complexes depending on the task at hand. This would explain the apparently conflicting results regarding the relative importance of the tail subdomains observed in various organisms.

To attain a more unified and cohesive picture, more research utilizing techniques from biophysics, biochemistry, genetics, and cell biology will have to be carried out. The generation of mice knockout strains and cell lines, more work on flies and nematodes, higher resolution crystallographic data and new techniques for analyzing single molecules or molecules in single cells (i.e., optical tweezers, CALI, etc.) will help answer the many unanswered questions. More research should be carried out to find the connection, at the signaling pathway level, between class I myosins and other classes of myosins. Is there cross-talk and coordination between them? Are they controlled through different or overlapping signaling elements? In addition, are there connections between class I myosins and non-myosin motor systems such as microtubule motors as has been suggested for class V myosins [Wu et al., 2000].

The existence of multiprotein actin binding complexes that contain class I myosins opens many avenues of investigation. Are they found in higher organisms? How do the components coordinate their functions? Are there different complexes in various organisms? For example, the myosin I SH3 domain is essential both for function and interaction with Bee1p/p116 in *S. cerevisiae* and *D. discoideum,* respectively, but is not essential in *A. nidulans myoA* and rat myr3. Do these myosins complex with different proteins that do not require an SH3 domain to bind with?

Answering these many questions will clearly provide future research with many interesting challenges.

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