Cell Motility and the Cytoskeleton 45:163–172 (2000)

Localization of Wild Type and Mutant Class I Myosin Proteins in *Aspergillus nidulans* Using GFP-Fusion Proteins

Roxanne A. Yamashita, Nir Osherov, and Gregory S. May*

Division of Pathology and Laboratory Medicine, University of Texas, M.D. Anderson Cancer Center, Houston

We have examined the distribution of MYOA, the class I myosin protein of the filamentous fungus *Aspergillus nidulans*, as a GFP fusion protein. Wild type GFP-MYOA expressed from the *myoA* promoter is able to rescue a conditional *myoA* null mutant. Growth of a strain expressing GFP-MYOA as the only class I myosin was approximately 50% that of a control strain, demonstrating that the fusion protein retains substantial myosin function. The distribution of the wild type GFP-MYOA fusion is enriched in growing hyphal tips and at sites of septum formation. In addition, we find that GFP-MYOA is also found in patches at the cell cortex. We have also investigated the effects of deletion or truncation mutations in the tail domain on MYOA localization. Mutant GFP-MYOA fusions that lacked either the C-terminal SH3 or a portion of the C-terminal proline-rich domain had subcellular distributions like wild type MYOA, consistent with their ability to complement a *myoA* null mutant. In contrast, mutants lacking all of the C-terminal proline-rich domain or the TH-1-like domain were mainly localized diffusely throughout the cytoplasm, but could less frequently be found in patches, and were unable to complement a *myoA* null mutant. The GFP-MYOA ΔIQ mutant was localized into large bright fluorescent patches in the cytoplasm. This mutant protein was subsequently found to be insoluble. Cell Motil. Cytoskeleton 45:163–172, 2000.

Key words: myosin; GFP; motility; fungi; endocytosis

INTRODUCTION

Myosins are mechanochemical enzymes that convert the stored chemical energy of ATP into directional movement along actin filaments [Mermall et al., 1998]. They are heteromeric proteins that consist of one or two heavy chain polypeptides and one or more light chain polypeptides that bind to the neck or hinge region of the heavy chain. The heavy chain has several domains consisting of the head or motor domain, the light chain binding domain or hinge region and a C-terminal tail domain. The motor domain is highly conserved among all myosins and consists of ATP- and actin-binding sites and contains the actin-activated ATPase activity.

Myosins play an important role in actin-dependent motility and the formation of actin rich structures in cells. Myosin proteins are divided into at least fifteen classes or

The first two authors contributed equally to the results of this work.

R.A. Yamashita's present address is Section of Cellular and Molecular Motility, Laboratory of Molecular Cardiology, National Heart, Lung and Blood Institute, National Institutes of Health, Rm 8N202, 10 Center Dr. MSC1762, Bethesda, MD 20892-1762.

Abbreviations: ATP, adenosine triphosphate; C-terminal, carboxyl terminal; GFP, green fluorescent protein; N-terminal, amino terminal; SH3, Src homology 3; TH, tail homology.

Contract grant sponsor: NIH; Contract grant number: GM53027.

*Correspondence to: Gregory S. May, Division of Pathology and Laboratory Medicine, Box 84, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.
E-mail: gsmay@mdanderson.org

Received 26 August 1999; accepted 5 November 1999

families based on sequences found in the motor domain and additional amino acid sequences appended either N-terminal or C-terminal to the motor domain [Cheney and Mooseker, 1992; Mermall et al., 1998]. Class I myosins are monomeric and have a short tail C-terminal to the motor domain [Korn and Hammer, 1990; Pollard et al., 1991]. There are numerous class I myosins and they can be subdivided based on sequence similarity. The amoeboid class I myosins were first described in *Acanthamoeba castellanii*, and are widely distributed phylogenetically. They have been identified in all eukaryotes examined thus far. Amoeboid class I myosins all share the same basic domain structure. C-terminal to the highly conserved motor domain is a light chain binding domain, also referred to as IQ motifs that bind calmodulin as the light chain in *A. nidulans*. C-terminal to the light chain binding domain is a TH1 domain that contains an ATP insensitive actin-binding site and is rich in basic amino acids, an SH3 domain, and a proline-rich domain that in the amoebae consists of a GPA/GPQ (glycine, proline, alanine/glutamine)-rich region. The in vivo importance of these domains to myosin I function is currently the subject of intensive investigation.

Our studies of the class I myosin of *A. nidulans*, MYOA, have resulted in some surprising discoveries. MYOA is required for protein secretion and polarized hyphal growth [McGoldrick et al., 1995]. Mutant MYOA protein lacking the highly conserved SH3 domain has wild type function [Osherov et al., 1998]. We have also found that a thirty amino acid proline-rich sequence just C-terminal of the SH3 domain was required for MYOA function [Osherov et al., 1998]. In addition, we have shown that an activating mutation and an inactivating mutation at the TEDS rule phosphorylation site in the motor domain have no effect on growth on solid medium and both mutations lead to equally poor growth in submerged culture [Yamashita and May, 1998a]. The activating mutation was also shown to lead to a constitutive activation of endocytosis, thus implicating MYOA in this process. Thus, in *A. nidulans* a single class I myosin has been shown to contribute to a variety of cellular processes requiring that the protein be recruited to different locations within the growing hyphae.

In contrast to our studies, the SH3 domain of the class I myosin in budding yeast (Myo5p) and *Dictyostelium discoideum* (myoB) is required for the function of these class I myosins [Anderson et al., 1998; Novak and Titus, 1998]. In budding yeast, the SH3 domain of Myo5p has been shown to bind to the protein verprolin and this binding is important in establishing cellular polarity [Anderson et al., 1998]. A mutant Myo5p that lacks the SH3 domain was able to correctly localize in cortical actin patches, although the SH3 domain alone was insufficient for localization. The inability of the SH3 domain to properly localize on its own and the fact that Myo5p lacking the SH3 domain correctly localizes implicates other domains of Myo5p for proper localization within the cell. Additional work examining the importance of the TEDS rule site for myoB function in *Dictyostelium discoideum* arrived at the conclusion that regulated phosphorylation at this site was essential to the function of myoB [Novak and Titus, 1998]. This is also in contrast to our observations that mutations in the TEDS rule phosphorylation site are viable, though it is clear that it is necessary for the fidelity of MYOA function in liquid culture because the phosphorylation site mutants display slower growth in liquid culture [Yamashita and May, 1998a].

The mechanism underlying how different class I myosin isoforms are localized to different places in the cell is not known. *Acanthamoeba castellani* has three class I myosin isoforms: IA, IB, and IC. Myosin IC is localized to the contractile vacuole and plasma membrane. Myosins IA and IB are associated with the plasma and intracellular membranes [Baines et al., 1992]. The differential localization of these three class I myosins suggests that a mechanism exists to target the different myosins to different places within the cell. Because MYOA is the only class I myosin in *A. nidulans* and it participates in a variety of cellular functions, this represents an excellent system in which to investigate the role of the different tail domains in the subcellular distribution of this protein. We report here that the single class I myosin, MYOA, of *A.nidulans* is localized to the growing hyphal tip and at sites of septum formation. Using wild type and mutant GFP-MYOA fusion proteins, we have investigated what domains are required for specific localization in the growing hyphae.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions

A. nidulans media used were yeast extract glucose (YAG) medium and minimal medium with either 1% dextrose (MM-glucose) or 0.1% glycerol (MMV-glycerol) as described previously [McGoldrick et al., 1995; Pontecorvo et al., 1953].

Plasmid Constructions

GFP [Fernandez-Abalos et al., 1998] was PCR amplified using oligonucleotide primers GFP-1 (5′AGAGGGTACCAATGGTGAGCAAGGGCGAG 3′) and GFP-2 (5′ CTCTCCATGGCCTTGTACAGCTCGTCCAT 3′), subsequently digested with *Kpn*I and *Nco*I, ligated into pBluescript in a three-way ligation with a 1.3kb *Nco*I-*Spe*I fragment of *myoA* containing an in-frame FLAG epitope upstream of the motor domain

region of *myoA*. The FLAG epitope and the *Nco*I site were introduced into *myoA* by PCR using the oligonucleotide myoA43 (ATTCACCATGGACTACAAGGACGATGACGACAAGGGTCACTCTCGACGTCCT). All plasmid constructs were sequence verified. Mutant tail domains were introduced into pAL5-GFP-MYOA by digesting the pGEM-MYOA motor and tail mutant constructs previously described [Osherov et al., 1998] with *Spe*I and *BamH*I and ligating into pAL5-GFP-MYOA from which the wild type *Spe*I-*Bam*HI fragment had been excised. The ΔC-terminus construct was first made blunt with Klenow at the *Bam*HI site prior to ligation. Wild type and mutant GFP-MYOA fusion proteins were expressed from the inducible *alcA* promoter from the vector pAL5 [Waring et al., 1989]. pAL5 has an approximately 3.7 kb genomic DNA fragment from the histone H2A locus, which is used to target integration at this locus by homologous recombination. This was used to insure that the fusion proteins being expressed were not recombined at *myoA* and might result in an incorrect interpretation of the results.

The *pyroA*-GFP-*myoA* plasmid was used to transform the CL3 strain [McGoldrick et al., 1995]. This plasmid was constructed by digesting pGEM-*myoA* [Osherov et al., 1998] with *Sma*I and *Spe*I and ligating with an *Asp*718 made blunt with Klenow and *Spe*I cut fragment of GFP-FLAG-*myoA*. This construct was then digested with *Sph*I and *Kpn*I to introduce the 2.5kb *pyro*A nutritional selective marker.

Strain Construction

pAL5-GFP-FLAG-*myo*A plasmids containing the different frame shift or deletion mutations were transformed into the GR5 strain. Primary transformants were purified three times on YAG medium. Twenty independent transformants of each construct were analyzed for GFP expression by fluorescence microscopy. Approximately 90% of the transformants expressed GFP-MYOA and three of each line were subsequently analyzed by Southern hybridization to identify those in which integration had occurred correctly.

The *pyro*A-GFP-*myoA* plasmid containing the wild type version of *myoA* driven by its endogenous promoter was transformed into the CL3 strain as previously described [Osherov et al., 1998]. The CL3 strain expresses *myoA* conditionally under the control of the *alcA* promoter. Primary transformants were purified three times on MM-glycerol medium that allows expression of the *alcA* driven wild type version of *myoA*.

Southern Analysis

Cultures of GFP expressing transformants were grown on YAG and genomic DNA was isolated as described previously [Osmani et al., 1987]. Southerns were probed with the histone H2A containing fragment of the plasmid pAL5.

Microscopy

Spores were germinated in either minimal medium containing glycerol, for intermediate activation of GFP-MYOA expression, or threonine for maximal activation. Cells were grown at room temperature to prevent bleaching of the GFP-MYOA. Microscopy was performed after 24 h (early germination) and 48 h (hyphal growth) on a Zeiss Axiophot microscope with a standard FITC filter set, using a Hamamatsu chilled 3CCD electronic camera. Images were collected and transferred to Adobe Photoshop V.4.0. For each strain, several fields containing 500–1,000 germlings were examined and representative fields were recorded.

Deconvolution microscopy images were collected on an Applied Precision Deltavision deconvolution microscope. For each image shown, 30 images were taken at 0.2-m steps and reconstructed using the Deltavision Deconvolution software (V.2.10).

Protein Gel Electrophoresis and Western Analysis

Cultures of all transformants were grown in liquid MM-glycerol, harvested, and pressed dry between paper towels. The cell mass was homogenized in a Tenbrok homogenizer in the buffer indicated in the figure legend. Protein samples were mixed 1:1 with 2× sample buffer and boiled for 5 min. Twenty micrograms of total cellular proteins were separated on a 6% SDS PAGE. The proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) by electroblotting. MYOA proteins were detected using an affinity purified polyclonal MYOA antibody raised against the C-terminal portion of MYOA [McGoldrick et al., 1995]. Peroxidase-conjugated goat anti-rabbit antibody was used as the secondary antibody. Chemiluminescent detection of the proteins by ECL (Amersham Corp., Arlington Heights, IL) was used to verify the presence of the various MYOA mutant proteins.

Growth Studies

To assess the functional ability of the introduced GFP-MYOA construct to complement growth in the absence of wild type MYOA, germlings were grown on rich YAG medium, under which the *alcA* promoter is repressed, allowing only the GFP-MYOA from the endogenous *myoA* promoter to be expressed. Growth assays were performed as described previously [May, 1989].

Genetic, Molecular Genetic, and Other Methods

Methods for the growth and genetic manipulation of *Aspergillus nidulans* have been described in detail elsewhere [Pontecorvo et al., 1953; Waring et al., 1989].

Similarly, those methods used in the manipulation of plasmids and molecular cloning have been described elsewhere [Waring et al., 1989].

RESULTS

GFP-MYOA Fusion Protein Rescues a *Myoa* Null and Is Correctly Localized

We previously showed by indirect immunofluoescence that MYOA was preferentially localized to growing hyphal tips in *A. nidulans* [McGoldrick et al., 1995]. In this study, we used a GFP-MYOA fusion protein to examine the cellular distribution of MYOA in living hyphae, but we first sought to determine if the GFP-MYOA fusion retained myosin function. GFP was fused to the amino terminus of MYOA and expressed from the *myoA* promoter after transformation into the *myoA* conditional null mutant strain CL3, as described previously for *myoA* deletion and truncation mutants [McGoldrick et al., 1995; Osherov et al., 1998]. Transformants were selected on MM-glycerol to allow for expression of wild type MYOA from the *alcA* promoter. They were then tested for growth on YAG medium, which represses expression from the *alcA* promoter and the wild type MYOA protein. Using this approach, we determined that the GFP-MYOA fusion protein was able to rescue the conditional null mutant phenotype of the CL3 strain on YAG medium (Fig. 1A). Though the GFP-MYOA fusion protein supported growth it was not able to support wild type rates of growth as determined by quantitative radial growth assays (Fig. 1B). All other aspects of hyphal morphology and asexual development were normal in these strains (Fig. 1A). We conclude, therefore, that GFP-MYOA retains sufficient function that we could use the GFP fusion proteins to examine wild type and mutant MYOA protein distribution in living hyphae.

Having established that wild type GFP-MYOA could support normal hyphal growth and asexual development, we next examined the distribution of wild type GFP-MYOA expressed from the *alcA* promoter construct in growing hyphae and early in germination when hyphal polarity is being established. Wild type GFP-MYOA was preferentially localized to growing hyphal tips (Fig. 2; see also Fig. 7), confirming our previous observations using indirect immunofluorescence [McGoldrick et al., 1995]. In addition, wild type GFP-MYOA was also seen in small patches throughout the cytoplasm enriched beneath the plasma membrane consistent with our observation that MYOA functions in endocytosis [Yamashita and May, 1998b].

Previously we showed that MYOA functions to establish polar hyphal growth because in the absence of MYOA there is no polar growth [McGoldrick et al., 1995]. Therefore, we wanted to look at the distribution of wild type GFP-MYOA early in germination. We performed this study using a deconvolution imaging system to provide better spatial resolution for the distribution of the protein. Early in spore germination, before germtube emergence GFP-MYOA appears primarily in brightly fluorescent cortical patches (Fig. 2A and B) that persist after germtube emergence (Fig. 2C and D). These corti-

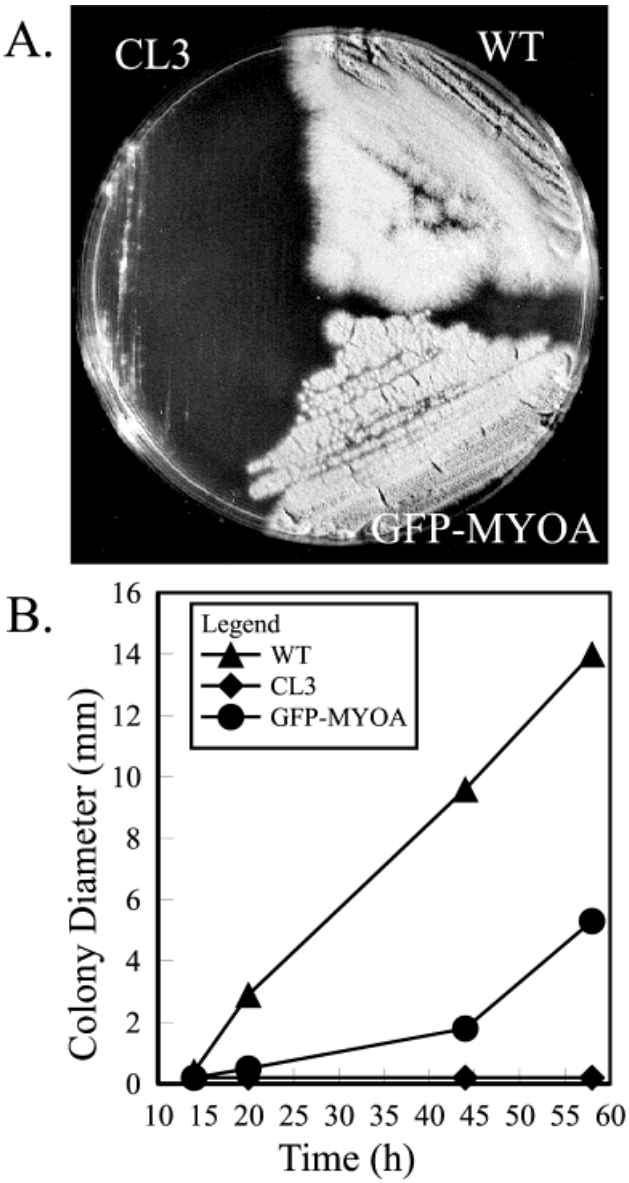

Fig. 1. GFP-MYOA fusion protein supports growth of *A. nidulans*. **A:** YAG plate containing three sectors streaked with spores from the conditional null *myoA* mutant strain CL3 (left half of the plate), CL3 transformed with wild type *myoA* (top right quadrant of the plate), and CL3 transformed with GFP-*myoA* fusion protein gene (lower right quadrent of the plate). **B:** Radial growth of individual colonies for the same strains shown in A, illustrating the quantitative difference in growth of the strain expressing the GFP-MYOA fusion protein compared to a wild type control strain.

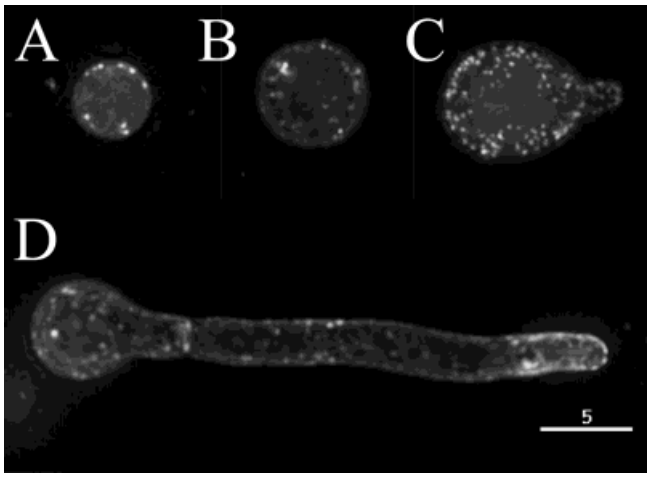

Fig. 2. Deconvolution images of *A. nidulans* spores (**A**) and very young germlings before (**B**) and at the time of germ tube emergence (**C**) and an older germling with a longer germ tube (**D**) all showing the GFP-MYOA primarily localized in cortical patches. Scale bar in D = 5 μm.

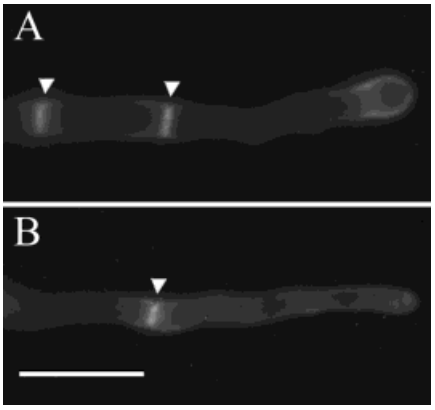

Fig. 3. GFP-MYOA localizes to septa in older hyphae. Two older hyphal tips (**A,B**) showing localization of GFP-MYOA at septa (marked with *white arrowheads*). Scale bar in B = 5 μm.

cally localized patches of GFP-MYOA can be seen along the entire length of the hyphae but clearly enriched at the growing tip and at the site of septal wall formation where it appears as a ring of MYOA (Fig. 2D).

In older hyphae with completed septal walls GFP-MYOA is frequently observed in abundance at septa (Fig. 3). Septation is the equivalent of cytokinesis in metazoan cells but in fungi cell division involves not only the formation of a contractile ring but the deposition of new wall material [Harris et al., 1994]. Since septation requires new wall formation, MYOA may function to carry vesicles containing wall precursors to these sites; therefore septation is probably related to the process of polarized hyphal growth at the tip.

Structure of Mutant Forms of MYOA and Southern Analysis of Transformants

Previously, we reported the construction of several *myoA* mutants using in frame deletions created by oligonucleotide-directed mutagenesis and restriction endonuclease sites to generate frame shift mutations [Osherov et al., 1998]. All of the mutations were made to create changes in the tail domains of MYOA. In the present study, we have used the same mutations to investigate their effect on the subcellular distribution of MYOA as GFP fusion proteins. The domains altered in each of the mutant forms of MYOA are depicted along with their phenotypic effect on the viability of the fungus (Fig. 4). In the current study, we have renamed the mutants FSXhoI and FSBamHI to ΔPro and and ΔC-terminus, respectively, to provide a more descriptive name for these two mutants.

Integration by homologous recombination of the plasmids used in this study can occur at either the H2A or *myoA* loci. Integration at either of these locations leads to predictable restriction endonuclease polymorphisms that can be used to identify the site of integration (Fig. 5). Genomic DNA from transformants was digested with *Xho*I and probed with a 3.7kb *Xba*I fragment of the H2A terminator from pAL5. Integration at the H2A locus would cause an increase in size from 3.7 kb (Fig. 6a, lane 1) in wild type cells to 7.7 and 9.3 kb for the endogenous and introduced sequences respectively (Fig. 5A). Surprisingly, this occurred in only one instance, the ΔPro mutant (Fig. 6a, lane 6). In all other cases, homologous integration of the plasmid occurred at the *myoA* locus, leaving the endogenous 3.7 kb H2A fragment unchanged in size and adding an additional 8.7 kb *myoA*-H2A fragment in the *myoA* locus (Figs. 5B and 6a, lanes 2–5).

The crossover between plasmid and chromosomal DNA leading to integration into the *myoA* locus can occur either upstream or downstream of the mutation. Those occurring downstream of the mutation would correct it, leading to the expression of *alcA* driven wild type *myoA* and mutant *myoA* lacking GFP driven by the endogenous *myoA* promoter. To identify these unwanted ''correcting'' integrations, the ΔTH1-like, ΔPro and ΔIQ transformants, that exhibit a marked growth impaired phenotype when solely expressed, were spread on YAG medium. Those that showed a mutant growth phenotype were discarded, as this was a result of expression of the mutant GFP-less *myoA* tail mutant off the endogenous *myoA* promoter. The ΔC-terminus mutant, which lacks an observable phenotype, was analyzed further by digestion with *Bam*HI and *Kpn*I, and probed with the H2A terminator fragment. Correct integration, upstream of the frame shift, results in an additional 8-kb fragment when compared with wild type cells (Fig. 6b).

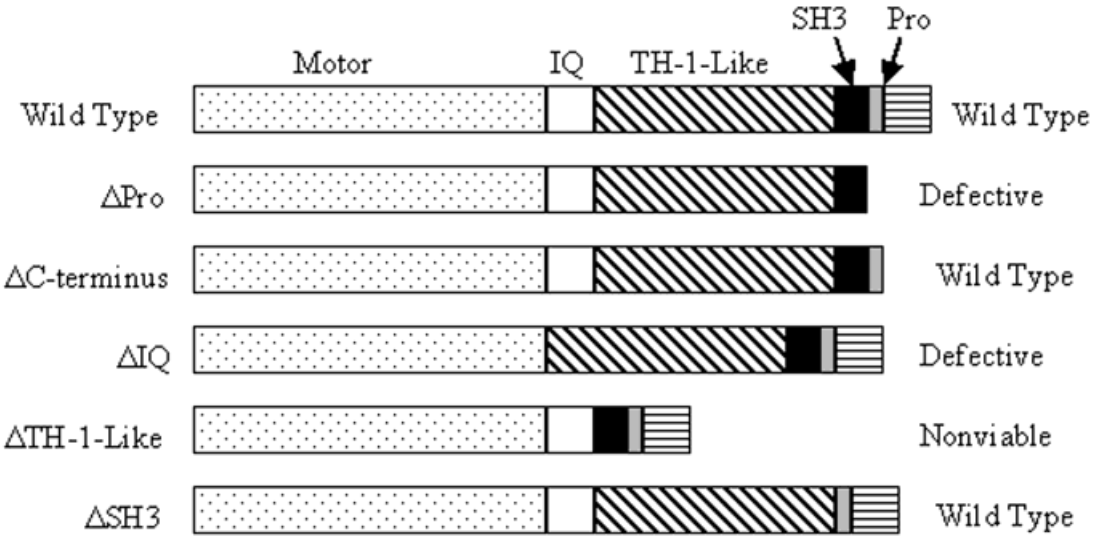

Fig. 4. Structure of wild type and mutant forms of MYOA showing the different domains of the protein and structural changes for each of the mutants. Motor domain sequences are stippled, IQ sequences are in white, the TH-1-like domain is diagonally hatched, the SH3 domain is in black, the proline-rich domain is gray, and the C-terminus is horizontally hatched. The phenotype resulting for each of the mutants is indicated to the right of each mutant form of the protein.

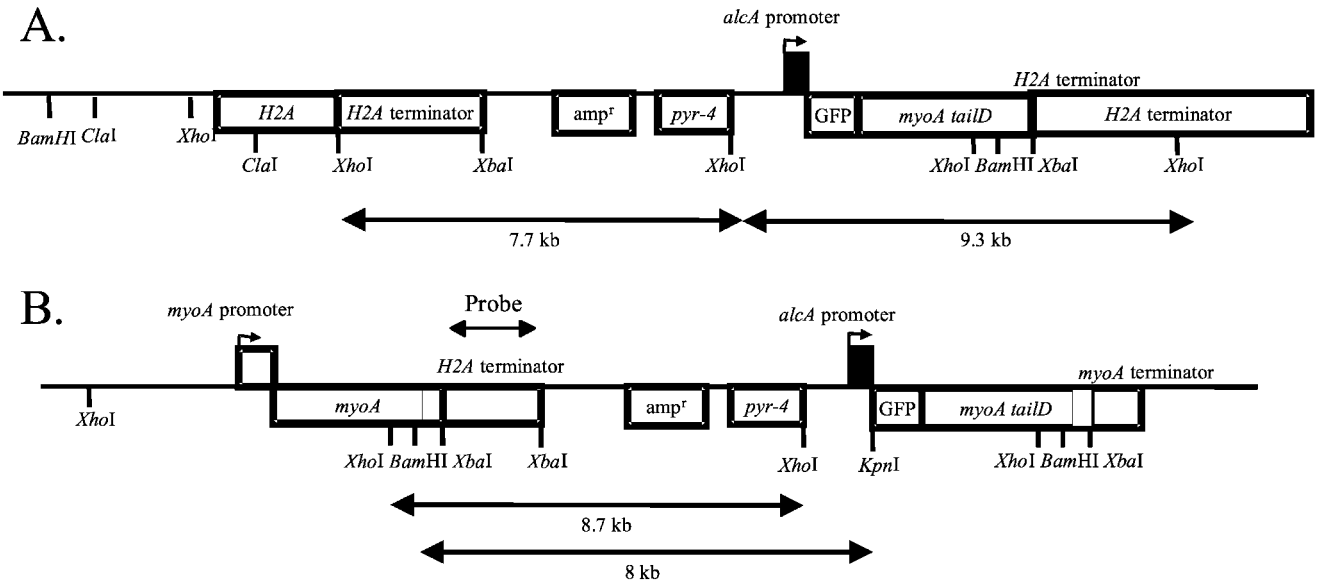

Fig. 5. Predicted restriction maps for integration of GFP-MYOA expression vector constructs at the histone H2A locus (**A**) or the *myoA* locus (**B**). The predicted sizes of bands hybridizing with the H2A fragment for integration at the two loci are indicated below each map. The segment of DNA used as a probe for the genomic shoutherns is indicated and labeled probe.

Localization of Mutant Forms of GFP-MYOA and Their Solubility

We previously reported that some frame shift and deletion mutants of MYOA were able to functionally complement the conditional null mutant *myoA* strain CL3 while other mutants could not, but we did not examine the cellular distribution of these mutant proteins [Osherov et al., 1998]. Using wild type and mutant GFP-MYOA fusion proteins, we have examined the distribution of the mutant proteins in vivo. As already described, GFP-MYOA is localized mainly in cortical patches that are enriched at the growing hyphal tip (Figs. 2D, 7A and B). Three mutants that fail to complement the conditional null mutant *myoA* strain CL3, ΔPro, ΔTH-1-like, and ΔIQ

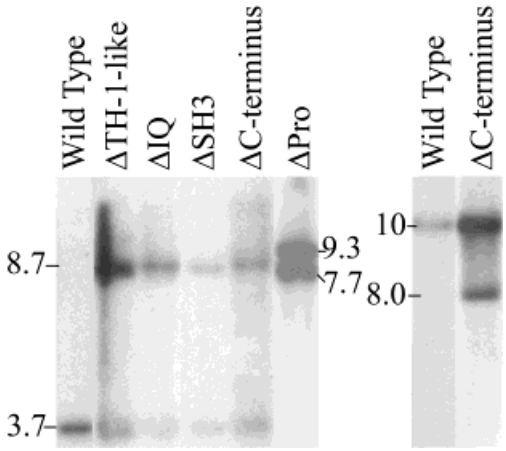

Fig. 6. Genomic Southerns showing the pattern of bands detected for each of the mutants. **a:** Integration of each of the mutants except ΔPro was at the *myoA* locus. ΔPro integrated at the histone H2A locus as indicated by the absence of the 3.7 kb band of hybridization. **b:** Southern showing integration of the ΔC-terminus mutant was correct.

exhibit a variety of aberrant localizations for GFP-MYOA. Both the ΔPro and ΔTH-1-like mutants result in a mostly diffuse localization of the mutant MYOA protein that is occasionally seen in large patches that are not at the cell cortex (Fig. 7C–F). In contrast to the mainly diffuse localization of GFP-MYOA seen in these two mutants, the ΔIQ GFP-MYOA mutant protein was observed to be in large cytoplasmic patches or aggregates that were not preferentially localized to the cell cortex (Fig. 7G and H). Thus, frame shift and deletion mutants that fail to complement a conditional null mutant *myoA* allele exhibit at least two different forms of mislocalization, either mostly as diffusely localized with some noncortical patches (ΔPro and ΔTH-1-like), or in large cytoplasmic particles (ΔIQ). In contrast to those mutants that fail to complement the conditional *myoA* null mutation of the strain CL3, those that did complement had subcellular distributions that were indistinguishable from that of wild type GFP-MYOA (Fig. 7I–L).

ΔIQ GFP-MYOA Mutant Protein Is Insoluble

The localization of the ΔIQ GFP-MYOA mutant protein to large particles in the cytoplasm led us to investigate the solubility of the mutant MYOA proteins. All of the mutant proteins except ΔIQ MYOA displayed solubility characteristics like that of wild type MYOA protein (data not shown). We therefore examined the solubility characteristics of the ΔIQ mutant protein in greater detail using Western blotting. Homogenates of hyphae expressing both wild type and the ΔIQ mutant MYOA proteins were fractionated by differential centrifugation and the presence of the two proteins in the pellet and supernatant was determined (Fig. 8). All of the ΔIQ MYOA mutant protein was found in the 14,000*g* pellet fraction under a variety of buffer conditions, including the presence of high salt (0.5M KCl), nonionic detergent (0.1% Triton X100), or 12 mM sodium pyrophosphate. We conclude that the large particles seen in the cytoplasm of ΔIQ GFP-MYOA are insoluble aggregates of the protein. This also explains the failure of the ΔIQ mutant protein to rescue the conditional *myoA* null mutation [Osherov et al., 1998].

DISCUSSION

MYOA, the class I myosin of *A. nidulans,* functions in hyphal morphogenesis, polar cell growth, protein secretion, endocytosis, and septum formation [McGoldrick et al., 1995; Osherov et al., 1998; Yamashita and May, 1998a]. Here we have shown that a GFP-MYOA fusion protein can be used to assess the distribution of wild type and mutant forms of MYOA. This represents the first time that GFP has been fused to an unconventional myosin and used to evaluate the cellular functions and distribution of the myosin. In this study, we have shown that the GFP-MYOA fusion protein is able to rescue a conditional null mutant *myoA* and displays a subcellular localization like that previously seen by immunofluorescence [McGoldrick et al., 1995]. We used this GFP-MYOA fusion to assess the distribution of mutant forms of MYOA.

The determinants for the subcellular distribution of class I myosins are unknown. In *A. castellanii* different class I myosin isoforms are localized to different subcellular compartments; therefore each of the isoforms must contain specific structural information that results in a different pattern of localization, but these have not been elucidated. In contrast, MYOA is the only class I myosin of *A. nidulans* and must therefore contain all the structural determinants necessary to recruit it to the various fungal cell compartments. In this study, we have shown that the ΔPro and ΔTH-I-like mutations fail to become properly localized. Failure to localize to specific sites in the cell explains why these two mutant forms of MYOA fail to rescue a conditional null mutant of *myoA* [Osherov et al., 1998]. This result indicates that the TH-1-like domain and the distal proline-rich region, deleted in the ΔPro mutant of MYOA, play a role in targeting MYOA to specific sites in the fungal cell. In contrast, the ΔIQ MYOA mutant protein was found in large aggregates throughout the cytoplasm. We have shown that these aggregates contain mutant MYOA protein that is insoluble, possibly explaining why this mutant protein is not able to rescue a conditional null *myoA* mutant.

The TH-1 domain of the *A. castellanii* myosin IC binds to membranes and synthetic lipid vesicles that contain acidic phospholipids, and may contribute to

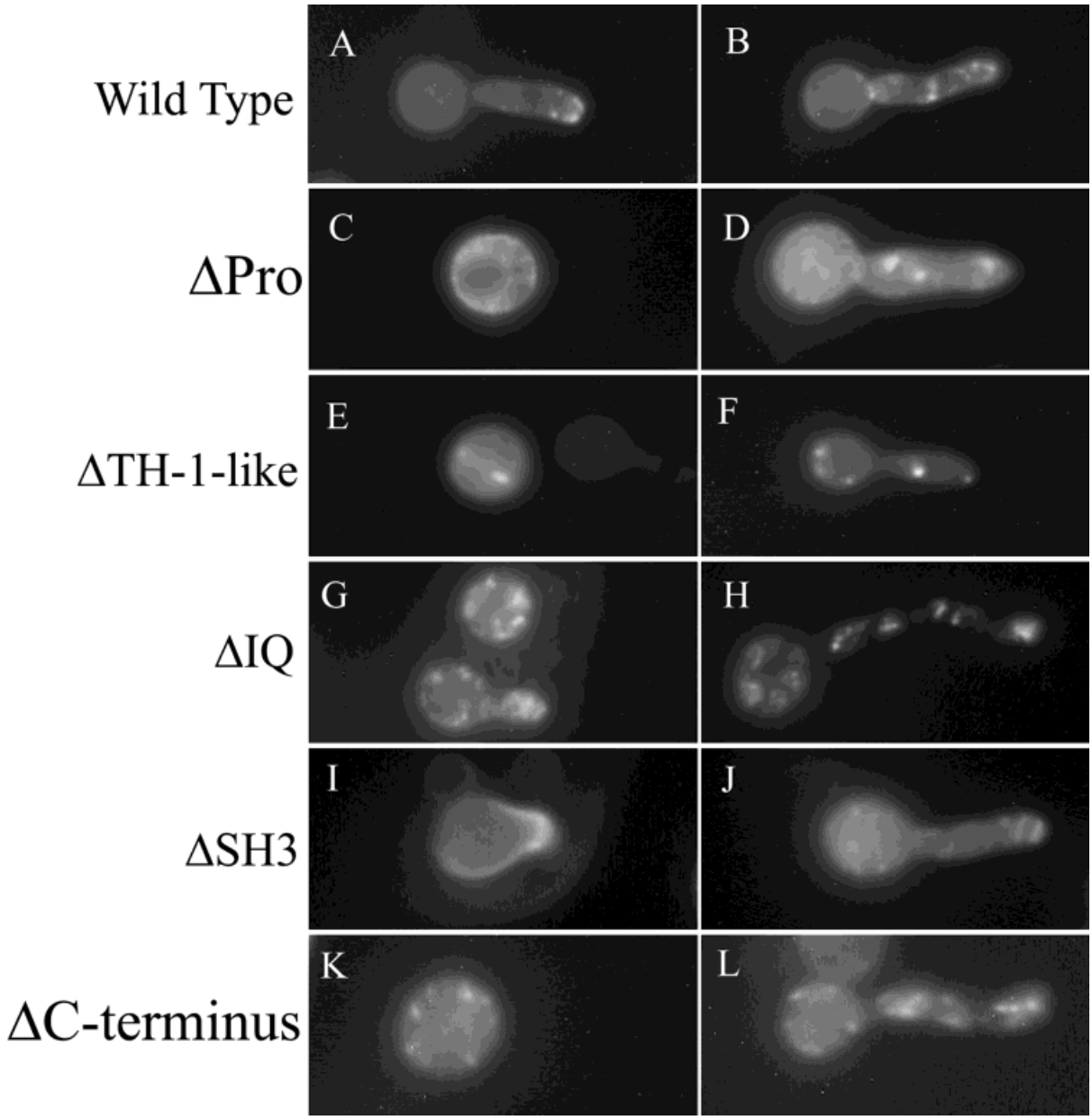

Fig. 7. Localization of wild type and mutant GFP-MYOA fusion proteins in germlings. Wild type GFP-MYOA is seen primarily at hyphal tips but is also seen as patches in the cytoplasm (**A,B**). The GFP-MYOA mutant proteins ΔPro (**C,D**) and ΔTH-1-like (**E,F**) are primarily seen as diffuse fluorescence throughout the cytoplasm and occasionally in noncortical patches. The GFP-MYOA ΔIQ mutant protein is localized to large patches throughout the cytoplasm (**G,H**). The GFP-MYOA ΔSH3 (**I,J**) and ΔC-terminus (**K,L**) mutant proteins display a distribution like wild type protein.

targeting this myosin to the contractile vacuole [Adams and Pollard, 1989a,b; Doberstein et al., 1993; Doberstein and Pollard, 1992]. While binding to acidic phospholipids can play a role in membrane targeting, specific binding of myosin IC to the contractile vacuole must be mediated through specific protein-protein interactions. In addition to binding to membranes with acidic phospholipids, the TH-1 domain of *A. castellanii* has been shown to have an ATP insensitive F-actin binding domain that would enable the myosin to slide actin filaments relative to one another. It is unlikely that this F-actin binding site plays a role in binding to the contractile vacuole membrane. This leaves open the possibility that the TH-1 domain may interact with other proteins that facilitate binding to specific membrane compartments. In this light, it is interesting to note that deletion of the TH-1-like domain

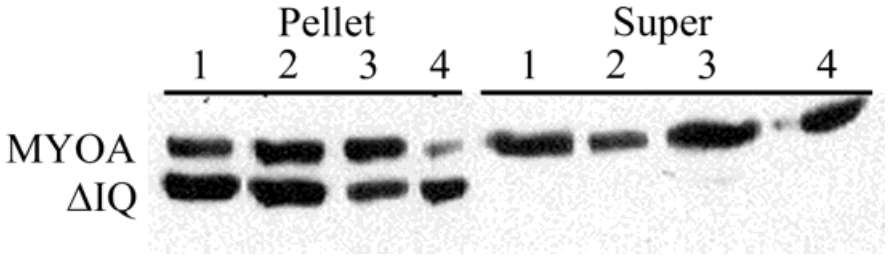

Fig. 8. ΔIQ MYOA mutant protein is insoluble. The CL 3 strain was transformed with a plasmid containing ΔIQ mutant *myoA* gene. This strain co-expresses wild type and mutant protein [Osherov et al., 1998]. Hyphal homogenates were prepared and centrifuged as desribed in Materials and Methods, the pellet and supernatant fractions were separated by SDS PAGE, and MYOA was detected by Western blotting [Osherov et al., 1998]. Homogenates were prepared in (1) 50 mM HEPES, pH 7.5, 75 mM KCl, 5 mM DTT and protease inhibitors (buffer A), (2) buffer A plus 12 mM $Na_4P_2O_7$, (3) buffer A plus 425 mM KCl, and (4) buffer A plus 425 mM KCl and 0.1% (v/v) Triton X-100.

from MYOA leads to a protein that is distributed diffusely throughout the cytoplasm. One reason for this may be that the TH-1-like domain plays a role in targeting this myosin to specific subcellular compartments through specific protein interactions. Resolution of this will require additional specific mutations in the TH-1-like domain of MYOA and biochemical studies of MYOA in vitro to determine if this protein binds acidic phospholipids and F-actin like that of the *A. castellanii* myosin IC.

The complementing ΔC-terminus and the non-complementing ΔPro mutants define a 30 amino acid proline-rich domain that is essential for MYOA function [Osherov et al., 1998]. In this study, we have found that the ΔPro mutant MYOA protein is not correctly localized in the fungal cell. Instead the protein is mainly diffusely localized throughout the cytoplasm, suggesting that the proline-rich 30 amino acids plays an essential role in MYOA function and localization. Proline-rich motifs like that of MYOA are known to bind to SH3 domains of other proteins [Mayer and Eck, 1995]. We, therefore, hypothesize that this proline-rich domain of MYOA binds to an SH3 domain of another protein contributing to the specific localization of MYOA. Curiously, the SH3 domain of MYOA is not essential for MYOA function [Osherov et al., 1998]. This is in contrast to work performed on the class I myosin myoB of *D. discoideum* where it was shown that the SH3 domain is essential for function [Novak and Titus, 1998]. Similarly, the SH3 domain of Myo5p of budding yeast has been shown to be necessary for proper localization and cell polarity [Anderson et al., 1998]. In addition, the SH3 domain of Myo5p was shown to bind to verprolin at a proline-rich sequence. Thus, while the SH3 domain of MYOA is not essential for function, a 30 amino acid proline-rich sequence immediately adjacent to the SH3 domain is essential for function. We predict that this proline-rich domain binds to an SH3 domain containing protein and leads to specific localization in the cell.

Our results suggest that the TH-1-like domain and the proline-rich domain play a role in targeting MYOA to specific sites in the fungal cell, presumably through specific protein-protein interactions. Further mutational studies will be necessary to precisely define the amino acid sequences in the TH-1-like domain that function in localizing MYOA to specific sites in the cell. Other than binding to acidic phospholipids by the *A. castellanii* myosin IC, our data suggest for the first time that the TH-1-like domain has a role targeting a class I myosin to a specific subcellular compartment in vivo. Using additional mutant GFP-MYOA fusion proteins, we will be able to identify those determinants in the TH-1-like domain that contribute to specific localization. It should then be possible to identify the proteins that bind to these domains as well.

ACKNOWLEDGMENTS

We thank John Mathew for his excellent technical support. This work was supported by a grant from the NIH GM53027 to G.S.M.

REFERENCES

Adams RJ, Pollard TD. 1989a. Binding of myosin I to membrane lipids. Nature 340: 565–568.

Adams RJ, Pollard TD. 1989b. Membrane-bound myosin-I provides new mechanisms in cell motility. Cell Motil Cytoskeleton 14:178–182.

Anderson B L, Boldogh I, Evangelista M, Boone C, Greene LA, Pon LA. 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, Brzeska H, Korn ED. 1992. Differential localization of *Acanthamoeba* myosin I isoforms. J Cell Biol 119:1193–1203.

Cheney RE, Mooseker MS. 1992. Unconventional myosins. Curr Opin Cell Biol 4:27–35.

Doberstein SK, Baines IC, Wiegand G, Korn ED, Pollard TD. 1993. Inhibition of contractile vacuole function in vivo by antibodies against myosin-I. Nature 365:841–843.

Doberstein S K, Pollard TD. 1992. Localization and specificity of the phospholipid and actin binding sites on the tail of *Acanthamoeba* myosin IC. J Cell Biol 117:1241–1249.

Fernandez-Abalos JM, Fox H, Pitt C, Wells B, Doonan JH. 1998.

Plant-adapted green fluorescent protein is a versatile vital reporter for gene expression, protein localization and mitosis in the filamentous fungus, *Aspergillus nidulans*. Mol Microbiol 27:121–130.

Harris SD, Morrell JL, Hamer JE. 1994. Identification and characterization of *Aspergillus nidulans* mutants defective in cytokinesis. Genetics 136:517–532.

Korn ED, Hammer JAd. 1990. Myosin I. Curr Opin Cell Biol 2:57–61.

May GS. 1989. The highly divergent β-tubulins of *Aspergillus nidulans* are functionally interchangeable. J Cell Biol 109:2267–2274.

Mayer BJ, Eck MJ. 1995. SH3 domains. Minding your p's and q's. Curr Biol 5:364–367.

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

Mermall V, Post PL, Mooseker MS. 1998. Unconventional myosins in cell movement, membrane traffic, and signal transduction. Science 279:527–533.

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

Osherov N, Yamashita RA, Chung YS, May GS. 1998. Structural requirements for in vivo myosin I function in *Aspergillus nidulans*. J Biol Chem 273:27017–27025.

Osmani SA, May GS, Morris NR. 1987. Regulation of the mRNA levels of *nimA*, a gene required for the G2-M transition in *Aspergillus nidulans*. J Cell Biol 104:1495–1504.

Pollard TD, Doberstein SK, Zot HG. 1991. Myosin-I. Annu Rev Physiol 53:653–681.

Pontecorvo G, Roper JA, Hemmons CM, MacDonald KD, Bufton AWJ. 1953. The Genetics of *Aspergillus nidulans*. Adv Genet 5:141–238.

Waring RB, May GS, Morris NR. 1989. Characterization of an inducible expression system in *Aspergillus nidulans* using *alcA* and tubulin-coding genes. Gene 79:119–130.

Yamashita RA, May GS. 1998a. Constitutive activation of endocytosis by mutation of *myoA*, the myosin I gene of *Aspergillus nidulans*. J Biol Chem 273:14644–14648.

Yamashita RA, May GS. 1998b. Motoring along the hyphae: molecular motors and the fungal cytoskeleton. Curr Opin Cell Biol 10:74–79.