

myoA of *Aspergillus nidulans* Encodes an Essential Myosin I Required for Secretion and Polarized Growth

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Abstract. We have identified and cloned a novel essential myosin I in *Aspergillus nidulans* called *myoA*. The 1,249-amino acid predicted polypeptide encoded by *myoA* is most similar to the amoeboid myosins I. Using affinity-purified antibodies against the unique myosin I carboxyl terminus, we have determined that MYOA is enriched at growing hyphal tips. Disruption of *myoA* by homologous recombination resulted in a diploid strain heterozygous for the *myoA* gene disruption. We can recover haploids with an intact *myoA* gene from these strains, but never haploids that are *myoA* disrupted. These data indicated that *myoA* encodes an essential myosin I, and this has allowed us to

use a unique approach to studying myosin I function. We have developed conditionally null *myoA* strains in which *myoA* expression is regulated by the *alcA* alcohol dehydrogenase promoter. A conditionally lethal strain germinated on inducing medium grows as wild type, displaying polarized growth by apical extension. However, growth of the same *myoA* mutant strain on repressing medium results in enlarged cells incapable of hyphal extension, and these cells eventually die. Under repressing conditions, this strain also displays reduced levels of secreted acid phosphatase. The mutant phenotype indicates that *myoA* plays a critical role in polarized growth and secretion.

MYOSINS are mechanochemical enzymes that bind to and move along actin filaments. All myosins have similar motor or head domains attached to structurally distinct tail domains and were originally classified into two groups, myosins I and myosins II. The best studied myosin II, or conventional myosin, is skeletal muscle myosin. Skeletal muscle myosins form dimers consisting of two globular heads attached to a coiled-coil tail, and they form thick bipolar filaments by ionic interactions between the tails of individual myosin molecules (Davis, 1988). Another example of a myosin II molecule is the nonmuscle myosin involved in contractile ring formation during cytokinesis (Fujiwara and Pollard, 1976; Mabuchi and Okuno, 1977; De Lozanne and Spudich, 1987; Knecht and Loomis, 1987). The myosins I, or unconventional myosins, originally included all myosins that were monomeric. However, because of the structural diversity of newly identified myosins, as many as eight myosin classes based on heavy chain structure have been described (Cheney et al., 1993; Goodson and Spudich, 1993). It is predicted that even more classes will emerge from this rapidly growing family of motor proteins.

Myosins I were first described in *Acanthamoeba castellanii* (Pollard and Korn, 1973), and they were discovered a

second time as the protein that functions as the radial link between the actin cytoskeleton and the plasma membrane in microvilli of intestinal brush-border cells (Collins and Borysenko, 1984). This microvillar myosin is now referred to as brush-border myosin I (BBMI)¹ (Mooseker and Coleman, 1989). Studies of myosin I function have historically focused on BBMI and amoeboid myosins I in *A. castellanii* and *Dictyostelium discoideum* (Pollard and Korn, 1973; Jung and Hammer, 1989). The structural features of the amoeboid myosins I are very similar. The amino-terminal motor domain is very similar to the globular head of conventional myosin that contains the actin-activated ATPase activity and that supports motility in *in vitro* assays. The tail domain contains three separate regions or tail homology domains (Horowitz and Hammer, 1990). Adjacent to the motor domain is tail homology domain 1 (TH-1), which has a net basic charge. The TH-1 domain of myosins I has been shown to bind to acidic phospholipid-containing vesicles and to plasma membranes treated with sodium hydroxide to strip them of peripheral membrane proteins (Adams and Pollard, 1989). The region responsible for membrane binding activity in *A. castellanii* myosin IC has been mapped between amino acids 701 and 888 (Doberstein and Pollard, 1992). The tail homology domain 2 (TH-2) of amoeboid myosins I is characterized as a region rich in the amino acids

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1. *Abbreviations used in this paper:* BBMI, brush border myosin I; SH3, src homology 3; TH-1, 2, and 3, tail homology domain 1, 2, and 3, respectively.

glycine, proline, and alanine or glutamine (GPA or GPQ domains). Tail homology domain 3 (TH-3) includes an SH3 (*src* homology 3) domain. This motif was originally described as a conserved, noncatalytic region in cytoplasmic tyrosine kinases such as p60^{c-src}, and it is found in many proteins involved with signal transduction including phospholipase C and the *Grb2* adapter protein (Mayer et al., 1993; Lowenstein et al., 1992). This domain is also found in proteins associated with membranes and actin cytoskeleton, and it includes the yeast actin-binding protein ABP1 and spectrin (Drubin et al., 1990; Wasenius et al., 1988). A chymotryptic fragment of *A. castellanii* myosin IA containing the GPA and SH3 domains has been shown to bind tightly to F-actin in an ATP-insensitive manner (Lynch et al., 1986). However, more recent analysis with glutathione-S-transferase fusion proteins containing the TH-2 and TH-3 domains of *D. discoideum* myosin IB and IC has shown that the TH-2 domain alone is sufficient for F-actin binding in a nucleotide-insensitive fashion (Rosenfeld and Renner, 1994; Jung and Hammer, 1994). This actin-binding site together with the nucleotide-sensitive site in the head enables myosin I to cross-link actin filaments (Fujisaki et al., 1985). Other myosins I that are lower in molecular weight than the originally reported myosins I have the basic membrane-binding domain, but they lack the GPA and SH3 domains. Examples of this class are the *myoA* and *myoE* gene products of *D. discoideum* and BBMI (Titus et al., 1989; Urrutia et al., 1993; Garcia et al., 1989). Thus, the differences in tail structure suggest distinct roles in cell behavior.

The molecular genetics available in *D. discoideum* has been used to generate null mutants by gene disruption and to determine the cellular function of myosins (Jung and Hammer, 1990; Titus et al., 1993; Jung et al., 1993). Disruption of myosin I genes by homologous recombination has resulted in cells with normal morphology and terminal development, but these cells display subtle defects in motility. *MyoA*⁻ and *MyoB*⁻ mutants of *D. discoideum* display a reduced average instantaneous velocity compared to wild-type cells. An increase in the formation of lateral pseudopodia and turning is seen, but the size of the lateral pseudopod is diminished. A reduced efficiency of chemotactic aggregation is evidenced by delayed cytoplasmic streaming, and they have a decreased rate of phagocytosis (Titus et al., 1993; Jung and Hammer, 1990). Similar disruption by homologous recombination experiments have been done in *Saccharomyces cerevisiae*. The results of disruption of a myosin I gene, *MYO3*, in this system has no obvious effects on cellular morphology or viability (Goodson, H. V., M. A. Titus, and J. A. Spudich, 1990. *J. Cell Biol.* 111:168a). To date, it has not been possible to determine myosins I function by gene disruption experiments. However, the presence of multiple isoforms (*D. discoideum* expresses at least five myosin I heavy chain isoforms) may allow for functional redundancy. Thus, disruption of multiple myosins in these systems will be necessary to determine the role of these motor proteins in cellular motility. The importance of one myosin isoform, *A. castellanii* myosin IC, has recently been shown by using antibodies that inhibit myosin IC function in vitro. This experiment indicates that myosin IC plays an essential role in contractile vacuole function in vivo (Doberstein et al., 1993).

In this report, we described a new member of the unconventional myosin I family, the *myoA* gene of *Aspergillus nidu-*

lans. The *myoA* gene product has sequence homology to other myosins I, particularly the amoeboid myosins I, but is most similar to *MYO3* of *S. cerevisiae*. We show by gene disruption that *myoA* is essential for cellular viability. In addition, we have constructed conditionally null *myoA* strains to assay the specific role played by this essential gene. By repressing *myoA* expression in these strains, we demonstrate that *myoA* is required for polarized growth and secretion. This is the first report using a genetic approach to study myosin I function that has led to the description of an essential myosin I isoform.

Materials and Methods

Aspergillus Strains, Culture Conditions, and Transformation

A. nidulans strains used in this study were GR5: *pyrG89*; *wA3*; *pyroA4* and R153: *wA3*; *pyroA4*. Strains were grown on complete medium supplemented with 5 mM uridine and 10 mM uracil (Käfer, 1977; Pontecorvo, 1953). For some experiments, the fungus was grown on 0.5% yeast extract, 1% glucose. Transformation of *A. nidulans* was performed as described previously (May, 1989).

Manipulation and Cloning of DNA

A 698-bp fragment of a presumptive *A. nidulans* myosin I was generated by PCR from an *A. nidulans* cDNA library. Two degenerate oligonucleotides were designed for use in PCR: (a) GAGAGAATTCGA(TC)AT(CT)-TACGG(TC)TTCGA, which encodes the sequence DIYGFEL and contains an engineered EcoRI restriction site at its 5' end; and (b) GAGAGATC-CACGTT(CT)TC(GA)AG(GA)AG(GA)CC, which encodes the complementary strand for the sequence GLEENV and contains a BamHI site at its 5' end. The sequences targeted by these primers are underlined on the *myoA* sequence and correspond to amino acids 423–429 for primer a and 644–649 for primer b (see Fig. 2). The restriction endonuclease sites were included to facilitate cloning of the PCR product. The amplified band was digested with EcoRI and BamHI, and it was subcloned into the plasmid Bluescript. Several independent clones were sequenced and found to contain identical nucleotide sequences. The deduced amino acid sequence was that which was expected for a myosin I protein.

The PCR-derived DNA fragment was subsequently used to screen an *A. nidulans* cDNA library in λ gt10 (Osmani et al., 1988). Five positive plaques were identified and purified. The inserts from each of these clones were subcloned into the plasmid Bluescript. Sequencing and restriction mapping of the clones provide that they were all derived from the same gene. The largest clone was fully sequenced and used to obtain DNA fragments used for gene disruption and antibody production (Fig. 1). The plasmid Δ 4*myoA* containing the NheI to KpnI internal fragment of *myoA* was constructed in the plasmid pRG3, and it was used to disrupt *myoA* (Waring et al., 1989). Using the EcoRV fragment of *myoA* cDNA head sequence as a probe, a complete genomic *myoA* clone was obtained by screening a λ pAn library (Holt and May, 1993). A SmaI to SpeI genomic clone fragment of *myoA* spanning 21 basepairs of 5' noncoding sequence and the first 363 amino acids of *myoA* protein sequence was cloned downstream of the *alcA* alcohol dehydrogenase promoter into the vector pAL3 (Waring et al., 1989). This plasmid was used to transform GR5 to uridine prototrophy. Transformants were selected on minimal medium containing glycerol as the sole carbon source, supplemented with vitamins, and made in 0.6 M KCl to osmotically stabilize the protoplasts (May et al., 1985). All DNA manipulations, library screening, and sequencing were as described previously (Ehinger et al., 1990). Preparation of genomic DNA for Southern analysis was as described previously (Osmani et al., 1987). Southern blots were hybridized and washed using high stringency conditions (May et al., 1985). The cDNA probe containing the EcoRV fragment of the *myoA* head sequence was labeled with [α -³²P]dCTP (Amersham Corp., Arlington Heights, IL) using the random primer method (Feinberg and Vogelstein, 1983).

Production and Purification of Antibodies

A PvuII to XbaI fragment from the cDNA that encodes a portion of the car-

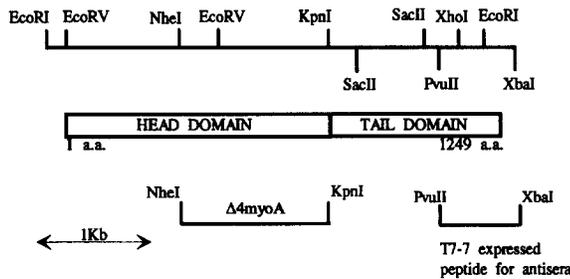


Figure 1. Partial restriction map derived from the cDNA of *myoA*. The extent of the open reading frame showing the myosin I head and tail domains is indicated below the map. Also shown below the map are the restriction fragments used in the construction of $\Delta 4myoA$ and *alcA-myoA*, both used for gene disruption, and the restriction fragment used to express protein from pT7-7 in *E. coli* for antisera production.

boxyl terminus was cloned into the expression vector pT7-7, and recombinant protein was expressed in the *Escherichia coli* host BL21 (Studier et al., 1990). The bacterially produced protein was fractionated by gel electrophoresis. After electrophoresis, the protein band was identified after a brief staining with 0.05% Coomassie blue (R250) in 50% methanol and 10% acetic acid. The band was cut from the gel placed in a dialysis bag, and the protein was electroeluted at 10 V/cm for 2 h. The recovered protein was mixed with Freund's adjuvant, and it was used to immunize rabbits.

For affinity purification of antibodies, the bacterially expressed protein was purified before coupling to Affigel-10 (Bio Rad Laboratories, Hercules, CA). Briefly, 8 liters of induced culture were harvested by centrifugation, resuspended in 25 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 1 mM EDTA with 1 mM PMSF, and frozen at -80°C . The cells were thawed on ice and sonicated. The particulate material in the suspension was pelleted at 100,000 g for 30 min, and the supernatant was brought to 45% saturation with ammonium sulfate. The protein precipitate was pelleted and dissolved in 50 mM sodium phosphate, pH 7.0, 0.1 mM EDTA, and 1 mM NaN_3 , and it was dialyzed against 4 liters of the same buffer. The dialyzed proteins were loaded onto a column containing Macro-Prep S (Bio Rad) support, and they were extensively washed with buffer. The bound protein was eluted in phosphate buffer plus 125 mM NaCl. The bacterially expressed protein was $\sim 90\%$ pure at this stage. The protein was dialyzed against 0.1 M sodium bicarbonate, pH 8.0, and coupled to Affigel-10 (Bio Rad) in bicarbonate buffer. Antibodies were affinity purified as described by Harlow and Lane (1988). Bound antibodies were eluted with 4 M MgCl_2 .

Protein Extraction, Gel Electrophoresis, and Immunoblotting. Mycelia were harvested by centrifugation or by filtration through Miracloth (Calbiochem-Behring Corp., La Jolla, CA), washed with ice-cold deionized water and briefly pressed dry. 200–500 mg dry wt mycelia were ground using a Ten Broek homogenizer (Fisher Scientific, Pittsburgh, PA) in the presence of extraction buffer, 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 5 mM EDTA, containing protease inhibitors (soybean trypsin inhibitor, aprotinin, leupeptin, L-1-tosylamide-2-phenylethyl chloromethyl ketone all at 10 $\mu\text{g}/\text{ml}$, 5 mM benzamidine, and 1 mM PMSF). In some experiments, the cells were broken open by vortexing in the presence of glass beads instead of using the Ten Broek homogenizer. Protein concentration was determined by the Bradford assay using bovine serum albumin as a standard (Bradford, 1976). Samples were mixed with sample buffer and electrophoresed on polyacrylamide gels. For immunoblot analysis, gels were transferred onto nylon and probed with affinity-purified anti-MYOA antisera at a 1:3,000 dilution, and then with alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1:1,000. The antibody complexes were visualized using a chemiluminescent detection system (Rad-Free; Schleicher & Schuell, Inc., Keene, NH).

MYOA Expression during Germination

R153 was grown at a density of 2×10^7 spores/ml in YG at 37°C , and 250 ml samples were taken from time 0 to 7 h. The samples were processed by centrifugation and resuspension in extraction buffer containing protease inhibitors as described above. The cells were broken open by vortexing in the presence of glass beads. 50 μg of protein from each sample was mixed with sample buffer and processed for electrophoresis as indicated above.

Cell Fractionation Assay

R153 was grown at 1×10^7 spores/ml in 50 ml of YG media for 16 h at 37°C . The mycelia were harvested by filtration through Miracloth, pressed dry, and weighed. The mycelia were then used directly or were protoplasted before homogenization (Yelton et al., 1984). 250 mg of mycelia was homogenized in 1 ml of extraction buffer, 25 mM Tris-HCl, pH 7.5, 0.5 M sucrose, and 0.1 mM EDTA with protease inhibitors. The whole-cell extract was spun at 1,000 g for 5 min. The supernatant, S1, was spun at 14,000 g for 15 min. The supernatant from this fraction, S2, was spun at 100,000 g for 1 h. The P2 and P3 pellets were resuspended in 100 μl of sample buffer. The 100,000 g supernatant, S3, was precipitated by the addition of trichloroacetic acid to 10%; the precipitate was pelleted and resuspended in 100 μl sample buffer. 75 μl of each sample was loaded on a 7.5% PAGE gel and processed as described above for immunodetection.

Growth Studies and Microscopy

Colony growth for the radial growth assay was initiated by plating conidia spores at a density of approximately five colony-forming units per plate onto triplicate plates. This ensures that each colony is formed by one or a few spores. Each point represents the average diameter of all colonies, except for strain CL3. The point on glucose medium represents a single colony that displayed growth at 96 h (see Fig. 8 a). This colony was abnormal and probably arose from a genomic rearrangement that restored *myoA* function. However, we have not tested this possibility.

Microscopic analysis was done by germinating R153 and CL3 conidia ($2 \times 10^6/\text{ml}$) at 37°C in YG media for the indicated times. The conidia settled onto coverslips present in the culture dishes. After the coverslips were rinsed in distilled water, the germlings were fixed in 3% glutaraldehyde in 10 mM potassium phosphate, pH 6.8, viewed by phase microscopy, and photographed.

For immunofluorescence, *A. nidulans* strain R153 was grown on glass coverslips, fixed, and processed as described (Denison et al., 1993), except that an extra permeabilization step in methanol at -20°C for 10 min was added. Affinity-purified anti-MYOA serum was used at a 1:20 dilution in PBS (2.7 mM KCl, 1.5 mM KH_2PO_4 , 0.137 M NaCl, and Na_2HPO_4 , pH 7.2). FITC-conjugated goat anti-rabbit IgG (Sigma Immunochemicals, St. Louis, MO) was used at a dilution of 1:100 to localize the rabbit antibodies. Staining for actin was performed as described (Harris et al., 1994).

Secreted Acid Phosphatase Assay

Secreted acid phosphatase activity was measured as described previously (Caddick and Arst, 1976) using 100 μl spent culture medium in 1 ml of 6-mM *p*-nitrophenol phosphate, 100 mM Pipes, and 2 mM EDTA, pH 6.0, at 37°C . Specific activity is expressed as nanomoles of *p*-nitrophenol liberated per milligram of dry weight of mycelia per minute at 37°C .

Materials. Restriction endonucleases and DNA-modifying enzymes were from New England Biolabs Inc. (Beverly, MA), Promega Biotech (Madison, WI), or Boehringer Mannheim Biochemicals (Indianapolis, IN), and they were used according to the manufacturer's instructions. Other reagents were from Sigma or Fisher Scientific.

Results

Cloning and Sequencing the *myoA* Heavy Chain cDNA

Using PCR, we obtained a specific hybridization probe for an *A. nidulans* myosin I and isolated cDNA and genomic clones. Sequence and restriction mapping indicated that all the cDNA clones isolated were derived from a single gene. The largest cDNA clone was completely sequenced, and the amino acid sequence of the myosin was deduced. The predicted amino acid sequence indicated that we had cloned a myosin I cDNA (Fig. 2). The structure of the predicted MYOA protein is most similar to that of MYO3 from *S. cerevisiae*, and it is similar to that of other amoeboid myosins I. The head domain of ~ 700 amino acids is highly homologous to the head region of all myosins, and the ~ 550 -amino acid carboxyl terminal domain shows limited homology to other myosins I. Immediately after the head domain is a re-

residues terminating with a tryptophan residue. The *MYO3* gene cloned from *S. cerevisiae* also terminates with this unusual motif (Goodson and Spudich, 1995).

The MYOA heavy chain sequence shows the greatest overall similarity to the heavy chain sequence of the *MYO3* gene from *S. cerevisiae*, as seen by dot matrix comparison (Fig. 3 A). A comparison of the derived MYOA heavy chain sequence to two amoeboid myosins I heavy chain sequences from *A. castellanii*, myosin IB and myosin IC, illustrates the high degree of sequence similarity in the head domain (Fig. 3, B and C). There is limited sequence similarity between the MYOA heavy chain sequence and either of the *A. castellanii* myosin I heavy chain sequences in the tail domain, with the exception of the SH3 domain. Another interesting feature of MYOA is the sequence that extends beyond the SH3 domain. This region differs from the GPA/GPQ domain described in other myosins, but it retains proline and alanine amino acids. In fact, the proline- and alanine-rich sequences resemble the sequence motifs of 3BP-1, which have been shown to bind the SH3 domain of the *Abl* tyrosine kinase protein (Cicchetti et al., 1992).

MYOA Is Present in Dormant Conidiospores

Using bacterially expressed recombinant protein, we have raised antibodies against the COOH-terminal portion of MYOA. The affinity-purified antisera specifically recognized a protein of the predicted molecular mass for MYOA, 138 kD, on an immunoblot of *A. nidulans*-soluble protein extract (Fig. 4 A, lane 2). Affinity-purified antisera preincubated with the recombinant protein against which it was raised failed to detect the MYOA protein band (Fig. 4 A, lane

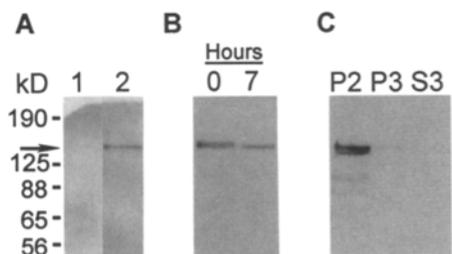


Figure 4. (A) Affinity-purified anti-MYOA antibodies recognize a band at the predicted molecular weight for the MYOA heavy chain in *A. nidulans* extracts. Shown is an immunoblot of R153 cell extract that was transferred onto nylon. In lane 1, the primary antibody was reacted with bacterially expressed MYOA fusion protein to compete for MYOA-specific antibodies before being used for detection. There are no bands present in this lane. Lane 2 is probed with the affinity-purified antibody. A single band of ~138 kD is present in this lane. (B) MYOA presence during conidiospore germination. Shown is an immunoblot of R153 cell extract from conidiospores that were germinated in YG medium at 37°C. The sample in lane 1 was collected at time 0, and the sample in lane 2 was collected after 7 h. When probed with affinity-purified anti-MYOA antisera, both lanes have a band of the predicted molecular weight for MYOA. (C) The subcellular location of MYOA was assessed by cell fractionation. Western analysis of the 14,000 g pellet (P2), the 100,000 g pellet (P3), and supernatant (S3), using the affinity-purified anti-MYOA antisera, indicates that MYOA is predominantly associated with the P2 fraction. There is some MYOA detectable in the P3 lane, but none in S3.

1). To learn more about MYOA regulation during spore germination, an immunoblot of soluble protein extract from conidiospores and growing germlings was probed with the affinity-purified anti-MYOA antisera. MYOA was detectable in germlings and, surprisingly, in dormant conidiospores (Fig. 4 B). The presence of MYOA in dormant spores suggest a role for this myosin in the early events of conidiospore germination. As we describe in a later section, this data is consistent with our studies of a conditionally lethal *myoA* mutant, which demonstrate that MYOA is required to establish polarized growth in germlings. Curiously, the migration of MYOA from conidiospores was slower than that observed from germlings. It is possible that the change in electrophoretic mobility was the result of a change in the phosphorylation state of MYOA in germlings versus conidiospores. Since other myosins I have been shown to be regulated by phosphorylation, this will be investigated for MYOA.

Immunolocalization of MYOA

To identify the cellular location of MYOA, germlings were stained with the affinity-purified anti-MYOA antisera. Actin localization was also analyzed in these germlings by staining with an antiactin monoclonal antibody. Indirect immunofluorescence of actin and MYOA (Fig. 5) indicate that actin is most highly concentrated at the growing tip of hyphae with punctate staining throughout the cytoplasm. MYOA also displays punctate staining at the tip (Fig. 5 B) and in the cytoplasm. The distribution of MYOA is similar to that observed for other myosin I isoforms, as antibodies raised against bovine adrenal myosin I stain highly motile, actin-rich cortical regions such as filopodia, lamellopodia, and ruffles in cultured cells (Wagner et al., 1992). Myosins I have been localized to the plasma membrane of locomoting *D. discoideum* and *A. castellanii* cells, particularly at the leading edge of advancing pseudopodia and in the phagocytic cups (Fukui et al., 1989; Baines et al., 1992). In *A. castellanii*, myosin IA is found almost exclusively in the cytoplasm

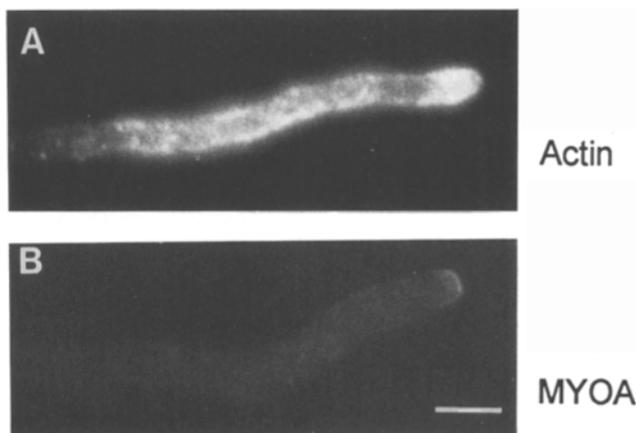


Figure 5. Immunolocalization of actin and MYOA in germlings. (A) An extending hypha with actin localization at the tip of the germ tube. Intense punctate staining is evident at the tip, but actin can also be seen as punctate spots extending throughout the cytoplasm of the cell. (B) MYOA is seen predominantly at the tip of the germ tube, and it is coincident with actin localization, but it stains with less intensity. Bar, 5 μ m.

associated with small vesicles and is also found in the cortex beneath phagocytic cups (Yonemura and Pollard, 1992; Baines et al., 1992).

The intracellular distribution of MYOA in *A. nidulans* was studied using a subcellular fractionation scheme modified from a protocol developed for *S. cerevisiae* (Ruohola and Ferro-Novick, 1987). Fractionation was performed on either whole mycelia or with protoplasts (Yelton et al., 1984). Almost all of the MYOA localized to the P2 fraction (14,000 g pellet), with some in the P3 pellet (100,000 g), indicating that it is largely membrane bound (Fig. 4 C). Studies in yeast using markers for the different organelles have shown that the P2 (or large particulate) fraction contains the endoplasmic reticulum, Golgi, mitochondria, and plasma membranes. The P3 is the microsomal pellet, and it contains mainly secretory vesicles (Walworth et al., 1989). The localization of MYOA to the fraction enriched in plasma membrane is consistent with the immunofluorescence localization data. However, the low amount of MYOA detected in P3 may not be very accurate since vesicle transport to the growing tip is very rapid and the corresponding abundance of vesicles is low.

myoA Encodes an Essential Myosin I

Gene disruption experiments have been used to identify genes essential for cellular viability in several organisms. We have disrupted the *myoA* gene in a diploid strain of *A. nidulans* by homologous recombination, and we confirmed it by genomic Southern analysis. We were unable to recover prototrophic haploids that would also be lacking *myoA* function (data not shown). This result suggested that *myoA* is essential in *A. nidulans*, or that strains lacking *myoA* function have a significant growth disadvantage. Using the following strategy, we have shown that *myoA* is an essential gene.

Because disrupted diploid strains are heterozygous for *myoA*, they cannot be used to study the phenotypic consequences of the absence of *myoA* function. An alternative strategy in which expression of the *myoA* gene could be regulated was used to address the question of MYOA function in *A. nidulans*. A *Sma*I to *Spe*I genomic clone fragment of *myoA* spanning 21 basepairs of 5' noncoding sequence and the first 363 amino acids of *myoA* protein sequence was cloned downstream of the *alcA* alcohol dehydrogenase promoter in the vector pAL3 (Waring et al., 1989). Integration of this plasmid by homologous recombination at the *myoA* locus resulted in strains that have the *myoA* gene situated immediately 3' to the *alcA* promoter (Fig. 6 A). These conditional *myoA* null mutant strains are dependent on the activity of the *alcA* promoter for transcription of *myoA*. Expression from the *alcA* promoter is regulated by the carbon source present in the medium. Primary transformants were selected for growth in minimal medium containing glycerol as the sole carbon source. Glycerol allows for a level of transcription from the *alcA* promoter that is intermediate between that seen on ethanol, which induces, or glucose-containing media, which represses *alcA* transcription (Fig. 6 B). This was done because over- or underexpression of MYOA might be inhibitory for growth. Transformants were then tested for growth on minimal media containing these different carbon sources. Strains that did not grow on glucose, but that grew on ethanol- or glycerol-containing media were selected and

analyzed by Southern (Fig. 6 C). All of the strains displayed the predicted genotype expected for conditional disruption of *myoA*. Two lanes on the Southern displayed multiple bands probably because of incomplete digestion by the restriction endonuclease.

The amount of MYOA present in soluble extracts of the *alcA-myoA* mutant strain, CL3 (Fig. 6 c, lane 10), grown in minimal media containing ethanol, glycerol, or glucose was assessed by Western analysis. MYOA was present when CL3 is grown on media with ethanol or glycerol present, but little or no MYOA was detected after growth in minimal media with glucose or in rich glucose media (Fig. 7, YG). When the *alcA* promoter was most strongly repressed in YG medium, CL3 contained little or no MYOA protein after incubation in this medium. MYOA was present in all of the extracts tested for the wild-type strain, R153.

Radial growth analysis provides a sensitive assay for deter-

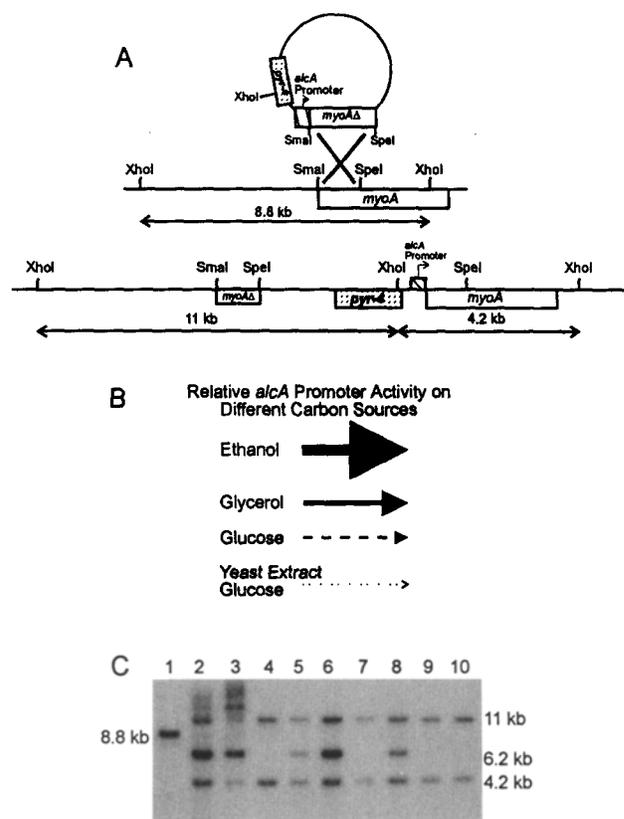


Figure 6. Construction of a conditionally null *myoA* mutant strain containing an inducible promoter. (A) The predicted integration and pattern of bands for *Xho*I-digested *myoA* DNA. (B) The effect of carbon source on *alcA* promoter activity. The level of transcription is maximal on ethanol-containing medium, is intermediate on glycerol, and is low when glucose is the carbon source provided. There is little or no transcription from the *alcA* promoter when strains are grown on rich medium (0.5% yeast extract, 1% glucose). (C) Genomic Southern blot for a wild-type nontransformed control (lane 1) and nine transformants that did not grow on glucose-containing medium (lanes 2–10). The expected pattern of bands (11.0 and 4.2 kb) is seen for all the transformants, with some exhibiting a third band (6.2 kb) predicted for a tandem integration of the plasmid. In lanes 2 and 3, multiple bands were detected that resulted from partial restriction enzyme digestion of the genomic DNA.

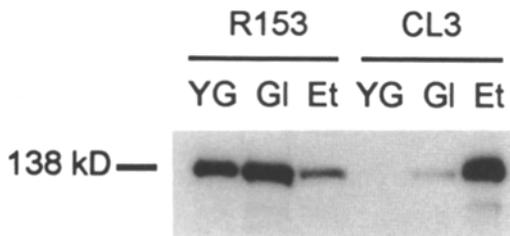


Figure 7. MYOA presence in R153 and CL3 strains grown on YG media (YG) or on minimal media with glucose (GI) or ethanol (Et) was tested. Shown is an immunoblot of R153- and CL3-soluble cell extracts after overnight growth in the indicated media. 50 μ g protein were loaded in each lane. Using the affinity-purified anti-MYOA antisera, a band of the predicted molecular weight for MYOA is detected in all lanes, except for the CL3 lane YG. The amount of MYOA present in the CL3 lane labeled GI is also reduced.

mining differences in growth rates of strains under various culture conditions. The radial growth of the *alcA-myoA* mutant strain, CL3, was compared to a wild-type strain, R153, on minimal media with ethanol, glycerol, or glucose as carbon sources (Fig. 8 a). Each measurement was the average of \sim 15 colonies for each strain analyzed, the exception being that only one CL3 colony grew on the glucose containing media and it was included in the figure. This colony also displayed sectoring, which indicates that genomic rearrangements had occurred. The CL3 strain grew just as well as R153 on ethanol- or glycerol-containing medium, and under these conditions, *myoA* was expressed. On minimal medium with glucose or in rich glucose medium (0.5% yeast extract, 1% glucose), *myoA* was transcriptionally repressed, and CL3 did not grow except for the aberrant colony previously mentioned. R153 had the fastest growth rate on this medium since glucose is a preferred carbon source. From this assay, it was clear that *myoA* was required for growth.

To examine the phenotype of an *alcA-myoA* mutant strain, CL3 conidia were germinated in YG medium, as was the control strain R153. After 11 h of growth in rich glucose medium R153, displayed normal growth (Fig. 8 b, panel A). The *alcA-myoA* strain exhibited no hyphal growth; instead, the conidiospores had swollen considerably and contained large vacuoles (Fig. 8 b, B). These germlings were unable to establish polarized hyphal growth, but they contained multiple nuclei and displayed a normal nuclear division cycle (data not shown). Extended incubation of CL3 did not result in a noticeable change in cellular morphology, and at the later time points of 17 and 34 h, the germlings became phase dark, suggesting that they were dead (Fig. 8 b, C and D).

Attempts to rescue the *alcA-myoA* mutant strain by plating on high osmolarity medium containing 0.6M KCl did not restore growth; instead, microscopic examination determined that many of the germlings had lysed (data not shown). Similar osmotic sensitivity has been observed in *S. cerevisiae* actin mutants and actin-binding protein mutants (Shortle, D., communication cited in Novick and Botstein, 1985; Chowdhury et al., 1992). More recently, it has been shown that when *A. castellanii* cells are loaded with antibodies that inhibited myosin IC function in vitro, the cells lysed when they were subjected to osmotic shock (Doberstein et al., 1993).

A. castellanii myosin IC has been previously localized to the contractile vacuole (Baines and Korn, 1990), and these results indicate an important role for the myosin I in the function of this organelle.

Acid Phosphatase Secretion Is Reduced in the CL3 Mutant Strain

Acid phosphatase secretion has been used as a marker for secretory mutant identification in *S. cerevisiae* (Novick et al., 1980). Furthermore, it has been shown that newly synthesized acid phosphatase secretion is polarized to the bud (Field and Schekman, 1980). In filamentous fungi, secretion is also polarized, and acid phosphatase has been localized to apical vesicles (Hill and Mullins, 1980). There are two possible explanations for the CL3 strain to not display polarized growth under repressing conditions does. One is that *myoA* is involved in the transport of essential components to the growing hyphal tip. This may involve transport through the cytoplasm to the tip, or MYOA may function in apical targeting of the vesicles. The other explanation is that MYOA is involved in the organization of the actin cytoskeleton at the growing tip of the cell. To test whether protein secretion is affected in the CL3 mutant strain, we compared secreted acid phosphatase levels with those of a wild-type strain (Table I). Acid phosphatase activity was measured in spent medium for cultures that were grown overnight in minimal media with the appropriate carbon source. When the mutant strain CL3 is grown in ethanol-containing medium, the level of secreted acid phosphatase is similar to that secreted by a wild-type strain (data not shown). In glucose medium, the levels of secreted acid phosphatase in the CL3 mutant strain are threefold lower than those in the wild-type strain, and they are similar to those seen in an acid phosphatase mutant *pacC14*. The impaired enzyme secretion in the mutant suggests that MYOA is involved in protein secretion, where it may act as a motor for vesicle transport.

Discussion

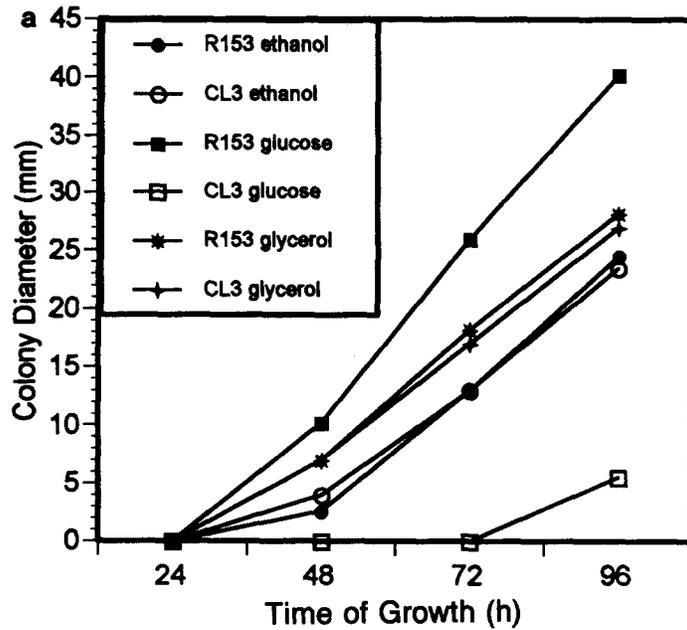
We have cloned a new unconventional myosin, *myoA*, from *A. nidulans*. The sequence of this myosin predicts it is a member of the amoeboid myosin I class and it is most similar to MYO3 from *S. cerevisiae*. The head domain closely resembles the head regions of other myosin I; however, the tail portion of MYOA has significant differences. The divergence of the tail from other myosin I sequences is characteristic of this class of myosins.

Disruption of myosins I in any system to date has indicated that they are not essential for growth or viability. Most of

Table I. Secretion of Acid Phosphatase by Wild-type and *alcA-myoA* Mutant *A. nidulans*

Strain	Acid phosphatase activity* Genotype	Glucose
R153	Wild type	58 \pm 14
<i>alcA-myoA</i>	<i>pyrG89</i>	19 \pm 16
<i>pacC14</i>	<i>choA1, ya2</i>	19 \pm 9

*Secreted acid phosphatase activity is expressed in nmoles *p*-nitrophenol liberated per milligram of dry mycelia per minute at 37°C. All cultures were grown in minimal medium with glucose at 37°C. The values are the mean \pm SD for three cultures assayed on different days.



b

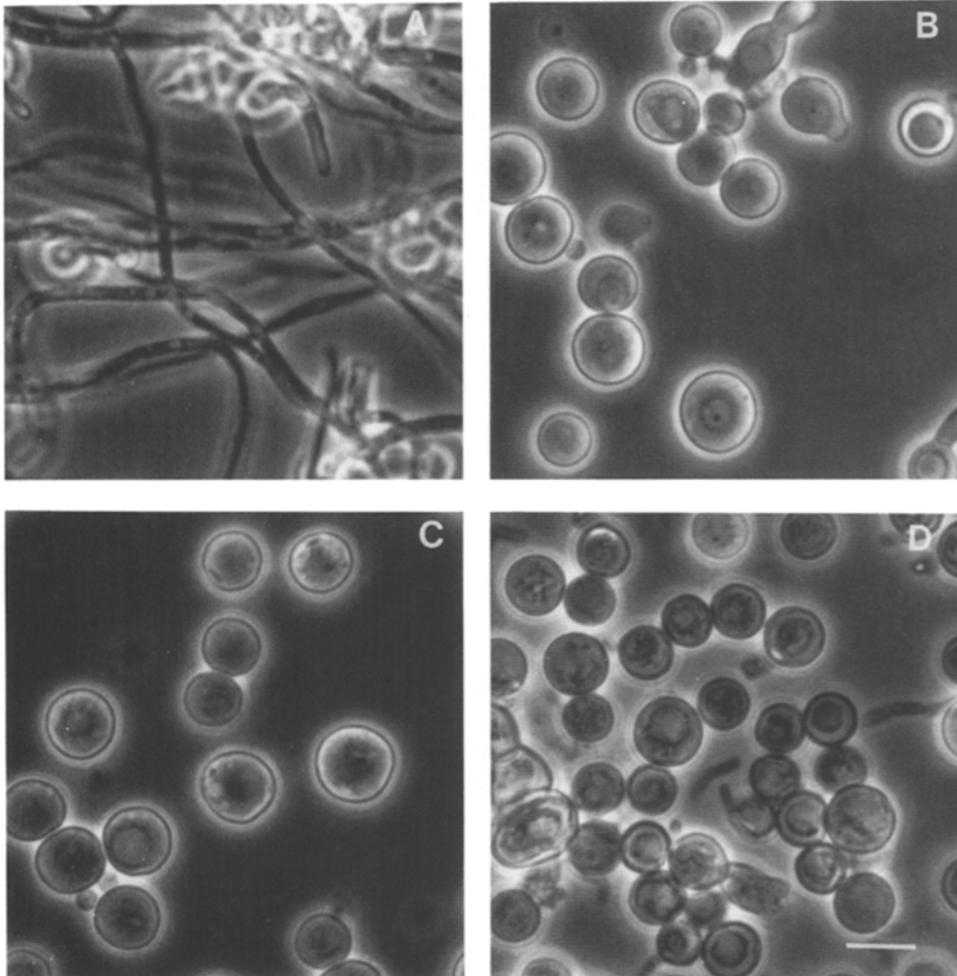


Figure 8. (a) Colony growth rates of a wild-type *A. nidulans* strain, R153, and the *alcA-myoA* strain, CL3, on minimal medium containing either ethanol, glucose, or glycerol as sole carbon source. (b) Cellular morphology of wild-type *A. nidulans* (A) and the CL3 mutant (B–D) strains grown on rich medium for 11 (A and B), 17.5 (C) or 34 (D) h. Bar in D, 10 μm .

these studies have been carried out in *D. discoideum* using homologous recombination to disrupt the myosin I gene. Disruption of unconventional myosins has resulted in cells with normal morphology and terminal development with only subtle defects (Jung and Hammer, 1990; Titus et al.,

1993; Jung et al., 1993). *D. discoideum* strains with null *MyoA*⁻ and *MyoB*⁻ mutant genes display a reduced average instantaneous velocity relative to wild-type cells. They also exhibit an increase in the formation of lateral pseudopodia, and they turn more frequently, but the size of the lateral pseu-

dopodia is diminished. A reduced efficiency of chemotactic aggregation is evidenced by delayed cytoplasmic streaming, and these strains have a decreased rate of phagocytosis (Titus et al., 1993; Jung and Hammer, 1990). Cells null for the *MyoD* gene of *D. discoideum* show no obvious phenotypic defects (Jung et al., 1993). However, the first indication of the importance of myosins I has come from the discovery that in *A. castellanii*, antibodies directed against myosin IC interfere with the function of the contractile vacuole in vivo, leading to overfilling of this organelle and cell lysis (Doberstein et al., 1993). This observation suggests an essential role for myosin IC in generation of the force required for contraction of the vacuole or in the transfer of water to the vacuole during refilling.

In this paper, we provide the first genetic evidence that a myosin I isoform performs an essential function. Gene disruption experiments in *A. nidulans* indicated the inability to maintain haploids with a disrupted *myoA* gene. The suitability of using *A. nidulans* for myosin I functional analysis is made evident by the identification of conditional *myoA* null mutant strains that allow regulation of *myoA* transcription. The result of repressing *myoA* transcription in these strains provide significant insight into the role played by *myoA* in *A. nidulans*. MYOA is required for hyphal tip emergence from germinating conidia. Interestingly, MYOA is found in the dormant spore, further suggesting it is required during the earliest events of germination. Reciprocal shift experiments in which the *alcA-myoA* mutant strains after 4 h are switched from inducing to repressing media show that many of these strains can germinate and grow relatively normally. If the strains are grown overnight in repressing medium and then shifted to inducing medium, many of the germings never extend germ tubes, and those that do often appear abnormal (data not shown). These data further indicate that MYOA is required for early events in polarized growth. There are two possible explanations for the mutant phenotype. One is that *myoA* functions to transport cellular and membrane components, cell wall precursors, and secreted enzymes required for growth along actin filaments, or that MYOA may be involved in attachment or fusion of transport vesicles at a specialized site on the plasma membrane. Alternatively, *myoA* could be involved in organizing the cortical actin cytoskeleton that leads to the formation of a polarized germling. MYOA is mainly localized at the growing tip of wild-type cells, although punctate staining is also seen throughout the cytoplasm. This type of distribution has also been observed for other myosin I isoforms. In *Acanthamoeba*, myosin IA is predominantly associated with small vesicles in the cytoplasm and with the cortex beneath phagocytic cups (Yonemura and Pollard, 1992; Baines et al., 1992). *Acanthamoeba* myosin IB and IC are associated with the plasma membrane and large vacuole membranes, but myosin IB alone is concentrated at phagocytic membranes, and myosin IC is the sole isoform associated with the contractile vacuole (Baines et al., 1992). In addition, myosins I are localized at the plasma membrane of locomoting *Dictyostelium* cells, particularly at the leading edge of advancing pseudopodia and in the phagocytic cups (Fukui et al., 1989). Cell fractionation experiments indicate that MYOA is mainly associated with the large particulate fraction, and that it is presumably associated with the plasma membrane. These data and the immunofluorescence localization of MYOA would

imply that *myoA* functions at the plasma membrane of the growing hyphal tip. The *alcA-myoA* mutant strains display reduced acid phosphatase secretion. Acid phosphatase is used as a marker enzyme in general secretion and so these results imply a role for myosins in vesicle transport of essential components to the growing tip rather than in actin cytoskeleton organization. The cell fractionation experiments do not accurately address MYOA association with vesicles since secretory vesicles are usually present in low abundance in wild-type cells since secretion is rapid and constitutive.

In those systems examined thus far, myosins I are coded for by an apparently functionally redundant multigene family. It was therefore a surprise when we determined that *myoA* was an essential gene in *A. nidulans*. This suggested that the myosin I gene family may not be as complex in *A. nidulans*. Using low stringency hybridization to a genomic Southern for *A. nidulans* and *S. cerevisiae*, we detected fewer bands in *A. nidulans* using a probe from the conserved head domain (data not shown). Using the same probe to screen a genomic library in λ phage at low stringency, we have been unable to clone other myosin genes from *A. nidulans*. These results suggest there are fewer myosin genes in *A. nidulans* than found in other organisms. However, our inability to detect additional myosin sequences does not prove that there are no additional myosin genes in *A. nidulans*.

The maintenance of cell wall integrity is particularly important at the tip of hyphal cells. Vesicles are rapidly transported to the growing tip, where they fuse with the membrane. Disruption of the composition of the membrane at the apical surface, e.g., choline deficiency, leads to cessation of polarized growth and multiple branching (Markham et al., 1993). In addition, the osmotic pressure must be maintained so that the cells retain their shape. The *alcA-myoA* mutants are osmotically sensitive, and secretion is much reduced. In addition, there is inappropriate hyphal branching if partial function of *myoA* is allowed by growth in minimal medium that contains glucose. This may indicate that in these mutants, the cell wall integrity is affected by the decreased transport of wall components to the tip. The evidence in this paper indicates a specific role for MYOA in secretion. However, it is not known whether MYOA is involved in both transport and secretion, or if it is acting as a receptor for vesicles at the apical membrane. Electron microscopic examination of the conditional lethal strains will address these questions and will provide further insight into the essential role of MYOA in polarized growth and secretion.

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