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Genetically engineered truncated myosin in *Dictyostelium*: The carboxyl-terminal regulatory domain is not required for the developmental cycle

(homologous recombination/mutant/slime mold)

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ABSTRACT **The study of engineered *Dictyostelium* mutants with altered or missing myosin has revealed the molecule to be essential both for cytokinesis and for completion of the complex *Dictyostelium* developmental cycle. To explore the biological role of the carboxyl-terminal portion of the myosin tail, we have created a *Dictyostelium* cell line bearing a mutation designated *myΔC34* in the myosin (*mhcA*) locus. This cell line produces a truncated myosin protein lacking the 34-kDa carboxyl terminus of the wild-type tail. Southern blots of the mutant cells show that the myosin gene was disrupted by homologous recombination of the transforming plasmid into the myosin locus. Based on *in vitro* studies of myosin functional domains, the 200-kDa truncated myosin was designed to include a domain important for assembly but to eliminate a domain important for threonine phosphorylation. The mutant cells are defective in cytokinesis, similar to those mutants that are either devoid of myosin (null cells) or contain a truncated 140-kDa myosin (*hmm* cells). However, unlike previous mutants, the cells carrying the *myΔC34* mutation are able to complete the *Dictyostelium* developmental cycle to form fruiting bodies. Thus a truncated 200-kDa myosin can substitute for native myosin to function in developing cells. These results demonstrate that the 34-kDa carboxyl terminus of myosin, which contributes regulated phosphorylation sites and 20% of the total length of the rod, is not required for the developmental cycle of *Dictyostelium*.**

The role of myosin in nonmuscle cells has recently undergone critical reevaluation. It was believed previously that myosin played a crucial role in many aspects of cellular motility, including endocytosis, cell movement, and cytokinesis. However, experiments that use either gene disruption or anti-sense methods to eliminate the large, conventional form of myosin in *Dictyostelium* have revealed only three cellular functions for which myosin is absolutely required. While endocytosis, intracellular organelle movement, and cellular locomotion continue in cells without myosin, cytokinesis, capping of cell surface receptors, and completion of the complex *Dictyostelium* developmental cycle are critically impaired (1–3).

In addition to the creation of cells that completely lack myosin, a second type of mutant has been created that contains only a partial myosin molecule. Instead of the native 240-kDa heavy chain, *Dictyostelium hmm* cells express *hmm*, a mutant protein in which the long tail of wild-type conventional myosin has been shortened by removal of 100 kDa of the carboxyl terminus (4). The *hmm* molecule includes the intact S-1 head region and the enzymatic domain of myosin molecules and, consequently, demonstrates many properties characteristic of wild-type myosin, such as binding to actin filaments and an actin-activated ATPase (5). However, despite retention of enzymatic activity by the *hmm* molecule *in vitro*, the phenotype of *hmm* cells is identical to that of null cells (cells devoid of myosin). These experiments suggest that the partial myosin molecule cannot contribute to myosin function in living cells; thus, the 100-kDa terminal portion of the tail appears to be critical for function *in vivo*.

The portion of the *Dictyostelium* myosin tail missing from *hmm* cells has been studied in experiments utilizing purified myosin tail segments. An internal 34-kDa domain has been shown to display assembly and solubility properties characteristic of myosin filaments (6). This assembly domain is adjacent to the 34-kDa carboxyl terminus of the tail, an important domain for phosphorylation by at least two distinct heavy-chain kinases that phosphorylate threonine residues (6–8). *In vitro* phosphorylation of these threonine residues has been shown to regulate the assembly of *Dictyostelium* myosin filaments (9, 10), which defines this 34-kDa carboxyl terminus as a regulatory domain. To understand the functional significance of this portion of the tail *in vivo*, we created and examined the phenotype of mutant cells (HS2218) that express a truncated 200-kDa myosin. These cells bear a mutation (designated *myΔC34*) in the myosin locus. The tail of the truncated myosin heavy chain, which is intermediate in length between the wild-type tail and the short *hmm* tail, was designed to contain the assembly domain of the tail but to eliminate the 34-kDa regulatory portion of the tail where *in vitro* threonine phosphorylation occurs. The phenotype of *Dictyostelium* cells carrying the *myΔC34* mutation would thus reveal the contribution of these domains of myosin to the physiology of living cells.

MATERIALS AND METHODS

General Methods. Cell lysates of wild-type and mutant cells were prepared and analyzed (5×10^4 cells per lane) on sodium dodecyl sulfate (SDS)/7.5% polyacrylamide gels as described (4). The gels were stained with Coomassie blue or transferred to nitrocellulose. Nitrocellulose transfers were incubated with a 1:1000 dilution of a polyclonal antiserum to *Dictyostelium* myosin (11) or with a dilution (15 μg of IgG/ml) of monoclonal antibodies My 4 or My 6 (12) or My 55 (7). The nitrocellulose filters were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG diluted 1:1000 (Bio-Rad) and then developed with 4-chloro-1-naphthol and hydrogen peroxide.

Dictyostelium DNA was prepared as described (2). Southern blots of *Dictyostelium* DNA were prepared by standard methods (13) and probed with fragments of the myosin gene

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Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.

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labeled with [α-^{32}P]dATP by using a random-primed DNA labeling kit [BMB (Indianapolis, IN)].

For nuclear staining, cells were fixed in 1% formaldehyde in methanol at −15°C for 5 min and stained with 4',6-diamidino-2-phenylindole (DAPI) as described (4). For study of the developmental cycle, vegetatively grown cells were collected by centrifugation, washed with starvation buffer (20 mM 2-(*N*-morpholino)ethanesulfonic acid/0.2 mM $CaCl_2$/2 mM $MgSO_4$, pH 6.8), and resuspended in the same buffer to a density of 2×10^7 cells per ml. Of this suspension, 9 ml was plated onto 1% agar plates made in starvation buffer and allowed to settle for 30 min. The excess liquid was aspirated, and plates were allowed to dry for 30 min, covered, and placed at 20°C in the dark to develop.

Construction of the Transformation Plasmid. The 3.7-kilobase (kb) *Bgl* II/*Xho* II fragment of the *mhcA* gene (14) was cloned into the *Bam*HI site of plasmid pTZ2.1 (2). Plasmid pTZ2.1 includes a transcription termination signal and a neomycin-resistance gene driven by the actin 15 promoter. The resultant plasmid contains the myosin gene fragment oriented with its 3' end immediately adjacent to the transcription termination signal of pTZ2.1. Only one *Bam*HI site, at the *Xho* II site at the 3' end of the myosin gene fragment, was preserved after ligation. A stop codon adjacent to the *Bam*HI site was put into the frame of the myosin gene by opening the *Bam*HI site, filling in with the Klenow fragment of DNA polymerase I, and religating the plasmid. The resultant plasmid, pMyΔC34, has two additional amino acids, arginine and serine, between the final *mhcA* codon (codon 1819) and the stop codon. The presence and frame of the stop codon were confirmed by sequencing the transformation plasmid.

Transformation of *Dictyostelium* Cells. Transformation of the pMyΔC34 plasmid into *Dictyostelium discoideum* strain Ax4 was carried out by forming a DNA-calcium phosphate precipitate and subjecting the cells to a glycerol shock as described by Knecht *et al.* (15); 1×10^7 cells received 10 μg of DNA. Control cells received 10 μg of pA15TX (16). Simultaneous selection and cloning of transformants took place as follows. The day after glycerol shock treatment, the cells were collected from the plastic plates, pelleted by centrifugation, and resuspended in 10 ml of HL-5 medium (23) containing 10 μg of G418 [Geneticin (GIBCO)] per ml (HL-5/G418). The cell suspension was mixed with 40 ml of 30°C molten low-gelling-temperature agarose [Seaplaque (FMC)] in HL-5/G418 (final concentration of agarose was 0.3%). Aliquots of this mixture (5 ml) were poured onto 100-mm plates containing a bottom layer of 35 ml of solid agar (0.35% Bacto-agar in HL-5/G418). The plates were left undisturbed at 20°C. After approximately 2–3 weeks, after colonies of transformants were clearly visible, each was picked with a Pasteur pipette. Each individual agarose plug containing a single colony was resuspended in 0.3 ml of HL-5/G418 and placed in a 24-well microtiter plate. When cells in the wells reached confluence, they were transferred and grown in 100-mm Petri dish plates containing HL-5/G418.

RESULTS

Design and Characterization of the Mutant. Homologous integration of a transforming plasmid into the *mhcA* locus was used to create a *Dictyostelium* cell that would substitute a truncated mutant myosin for wild-type myosin. To this end, a transformation plasmid was constructed containing an internal 3.7-kb segment of the *mhcA* gene and a G418-resistance gene (see *Materials and Methods*). This plasmid contained a termination codon introduced after codon 1819 of the *mhcA* sequence (14). Cells in which the *mhcA* locus was disrupted by the transforming plasmid would transcribe an approximately 200-kDa mutant myosin in place of the endogenous 240-kDa wild-type myosin.

Twenty-one independent clonal transformants were selected by resistance to G418 and were screened with a polyclonal anti-*Dictyostelium* myosin antiserum by immunoblot analysis. In cell lysates of 18 clones, the anti-myosin antiserum reacted with a single 240-kDa band that comigrated with wild-type myosin. However, three clones showed reactivity of a single band migrating at the position of approximately 200 kDa, the molecular mass expected for the mutant myosin (Fig. 1). Comparison of wild-type and mutant cell lysates analyzed on Coomassie blue-stained polyacrylamide gels revealed that the protein levels of the truncated myosin relative to those of wild-type myosin were diminished by at least 50% (Fig. 1*A*, lanes 1 and 2). This suggested that either the mRNA or the protein of the truncated myosin was less stable than that of wild-type myosin.

Several monoclonal antibodies previously mapped to defined locations on the *Dictyostelium* myosin molecule were used to verify epitopes on the mutant myosin consistent with a molecule that lacked the 34-kDa carboxyl terminus of the tail (Fig. 1). Both wild-type myosin and the mutant myosin reacted with My 6, a monoclonal antibody mapped to the head–tail junction, as well as My 4, a monoclonal antibody mapped to 135 nm from the junction along the length of the tail. My 4 binds to an epitope that lies immediately prior to the introduced termination codon (6, 17). In contrast, only wild-type myosin, and not the mutant myosin, reacted with My 55, a monoclonal antibody mapped to the carboxyl-terminal tip of the *Dictyostelium* myosin tail (7). Thus, as predicted, the mutant myosin reacted with monoclonal antibodies that bind to locations within the 200-kDa amino terminus of the native myosin heavy chain and failed to react with a monoclonal antibody whose epitope is within the 34-kDa carboxyl terminus of the tail.

Southern blot analysis confirmed that mutant cell lines encoding the 200-kDa protein arose from homologous integration of the plasmid into the wild-type *mhcA* gene locus. A probe generated from the DNA encoding the 34-kDa carboxyl terminus of the wild-type myosin heavy chain (probe I, Fig. 2 *C* and *D*) was used to hybridize to DNA from wild-type Ax4 or HS2218 cells. This probe hybridized to an 11.9-kb *Bcl* I/*Sal* I fragment, an 8.8-kb *Bcl* I/*Xba* I fragment, and to a 4.8-kb *Bgl* II/*Sal* I fragment in Ax4 cells (Fig. 2*A*, lanes 1–3,

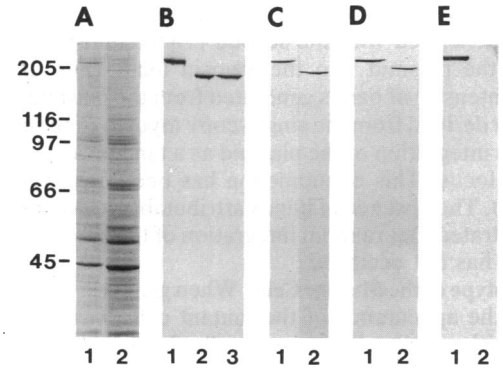

FIG. 1. Analysis of myosin in mutant cells. Shown are lysates derived from wild-type Ax4 cells (lanes 1), HS2218 cells carrying the *myΔC34* mutation (lanes 2), and cells germinated from HS2218 spores (lane 3). (*A*) SDS/7.5% polyacrylamide gel stained with Coomassie blue. (*B–E*) Immunoblots of duplicate gels stained with anti-*Dictyostelium* myosin antibodies; (*B*) polyclonal anti-*Dictyostelium* myosin antiserum; (*C*) My 6, monoclonal antibody mapped to the head–tail junction; (*D*) My 4, monoclonal antibody mapped to 135 nm from the head–tail junction; and (*E*) My 55, monoclonal antibody mapped to the tip of the tail. Molecular mass markers (kDa) are shown on the left.

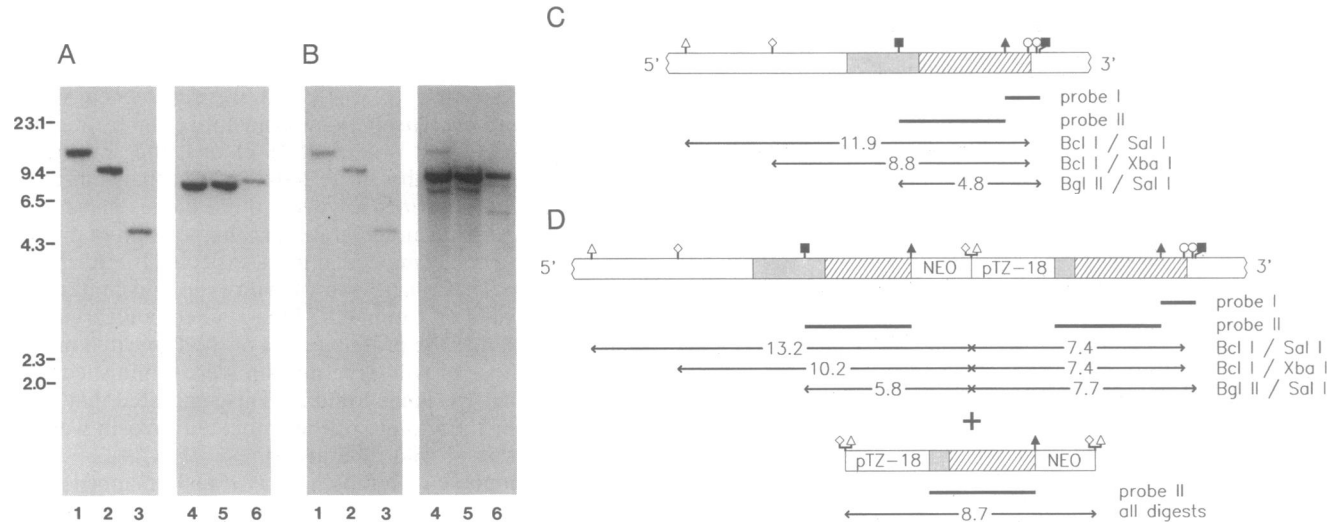

FIG. 2. Southern blot analysis of mutant cells. Shown are Southern blots of DNA derived from the parental strain Ax4 cells and mutant HS2218 cells probed with the 1.1-kb *Xho* II–*Bgl* II fragment (*A*) or the 3.7-kb *Bgl* II/*Xho* II fragment (*B*) of the *mhcA* gene. Lanes: 1 and 4, *Bcl* I/*Sal* I digest; 2 and 5, *Bcl* I/*Xba* I digest; 3 and 6, *Bgl* II/*Sal* I digest. Molecular size markers (in kb) are shown on the left. Restriction maps of fragments generated from Ax4 DNA (*C*) and HS2218 DNA (*D*) are shown. Probe I indicates the position of the 1.1-kb *Xho* II/*Bgl* II fragment and probe II indicates the position of the 3.7-kb *Bgl* II/Xho II fragment. Open bars represent 5′ and 3′ flanking DNA, stippled bars represent the coding region of myosin S-1 head, and hatched bars represent the coding region from the myosin tail; the position of plasmid pTZ-18 and the neomycin-resistance gene (NEO) are indicated. ○, *Bcl* I; ■, *Bgl* II; △, *Sal* I; ◇, *Xba* I; ▲, the position of codon 1819 of the *mhcA* sequence. The arrows indicate the size in kb of the respective fragments.

respectively), as expected from the *mhcA* gene map (Fig. 2*C*). In contrast, the same probe hybridized to a 7.4-kb *Bcl* I/*Sal* I fragment, a 7.4-kb *Bcl* I/*Xba* I fragment, and a 7.7-kb *Bgl* II/*Sal* I fragment in the mutant HS2218 cells (Fig. 2*A*, lanes 4–6). This pattern is predicted for the disruption of the *mhcA* gene by the transforming plasmid (Fig. 2*D*). The nitrocellulose blots were stripped and treated with a probe generated from the portion of the myosin gene contained within the integrating plasmid (probe II, Fig. 2 *C* and *D*). Probe II hybridized to an 11.9-kb *Bcl* I/*Sal* I fragment, an 8.8-kb *Bcl* I/*Xba* I fragment, and a 4.8-kb *Bgl* II/*Sal* I fragment in DNA from Ax4 cells (Fig. 2*B*, lanes 1–3). However, this same probe hybridized to 13.2- and 7.4-kb *Bcl* I/*Sal* I fragments, 10.2- and 7.4-kb *Bcl* I/*Xba* I fragments, and 5.8- and 7.7-kb *Bgl* II/*Sal* I fragments in DNA derived from HS2218 cells. In addition to these, an 8.7-kb fragment derived from the plasmid was common to all digests. The pattern generated with the second probe confirmed integration of the plasmid into the myosin locus (Fig. 2*D*). The darker intensity of bands generated from the plasmid relative to bands derived from the single-copy myosin gene suggested multiple integration of the plasmid as a tandem array into the myosin locus. This phenomenon has been observed previously (4). The absence of bands attributable to other loci also demonstrated that random integration of the plasmid into the genome has not occurred.

Phenotype of the Mutant Cells. When grown in nutrient-rich media, the appearance of the mutant cells was identical to that of either null cells or *hmm* cells. Inspection of the cells in the microscope revealed many large cells, which often displayed aberrant shapes. Video microscopy demonstrated that cells were capable of movement along a substratum and exhibited intracellular movement of organelles (data not shown). Although the parental Ax4 strain divides readily when grown in suspension cultures, the cells carrying the myΔC34 mutation grew very large and eventually died after several days in suspension cultures. Staining with DAPI, a dye specific for nucleic acids, revealed that mutant cells accumulated numerous nuclei (Fig. 3), indicating a defect in cytokinesis. Mutant cells were maintained on a solid substratum, where they appeared to divide by occasional pinching off of cellular fragments.

Surprisingly, the phenotype of the mutant HS2218 cells placed in starvation media was not identical to that of the null cells or *hmm* cells. Unlike the latter mutants, which are not able to progress in the developmental cycle beyond the aggregation stage, the cells carrying the *myΔC34* mutation completed all stages of the *Dictyostelium* developmental cycle. When placed on a solid substratum in starvation buffer, cells were observed to stream toward an aggregation center and to form multicellular slugs which subsequently formed fruiting bodies (Fig. 4). HS2218 cells were observed to undergo all stages of the developmental cycle; however, comparison of HS2218 cells with wild-type cells revealed some differences. Streams of HS2218 cells converging toward an aggregation center frequently had a broken appearance (Fig. 4*A*), whereas streams of wild-type cells were more continuous and uninterrupted. HS2218 aggregates were clearly able to form pseudoplasmodia (Fig. 4*B*); however, subsequent actively migrating slugs were seen with less frequency than in wild-type populations. This observation suggests that many mature fruiting bodies from HS2218 cells arose from pseudoplasmodia that had not migrated. In addition, the mutant cells took several hours longer to complete development than did wild-type cells.

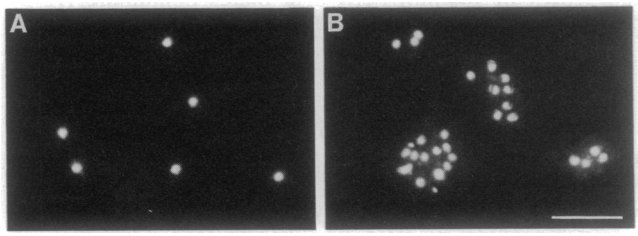

FIG. 3. Cells carrying the *myΔC34* mutation are defective in cytokinesis. Cells were allowed to attach to coverslips for 15 min and subsequently were fixed and stained with DAPI, a dye for nucleic acids. (*A*) Six mononucleate wild-type cells. (*B*) Four multinucleate HS2218 cells. (Bar = 25 μm.)

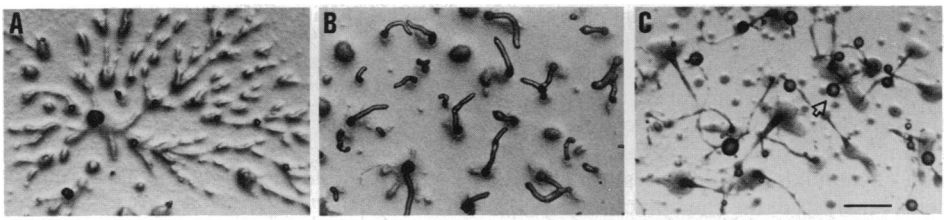

FIG. 4. Stages in the developmental cycle of cells carrying the *myΔC34* mutation. Mutant HS2218 cells were suspended in starvation buffer and then allowed to attach to agar starvation plates. Examination with a dissection microscope of the plates throughout development revealed cells in successive stages of aggregation (*A*), formation of early pseudoplasmodia (*B*), and formation of mature fruiting bodies (*C*). The arrow in *C* indicates a fruiting body. (Bar = 1 mm.)

To ensure that the ability of the mutant cells to complete development was not due to loss of the integrated plasmid and consequent recovery of full-length myosin, spores were collected and germinated in nutrient-rich medium. Lysates from the resultant cells were then analyzed on an immunoblot with a polyclonal anti-myosin antiserum. Of nine sori [each sorus, or ball of spores on a cellulose stalk (Fig. 4), generally contains about 10^5 spores] collected and germinated individually without drug selection, six contained only a single 200-kDa myosin (Fig. 1*B*, lane 3), while three contained a mixture of wild-type 240-kDa myosin and mutant 200-kDa myosin. The presence of the 240-kDa myosin in three sori is probably due to excision and loss of the integrated plasmid. This loss is expected to occur if mutant cells are germinated and grown without drug selection. All sori germinated in the presence of drug selection contained only the 200-kDa myosin. All cells derived from sori that contained only the mutant myosin remained unable to undergo cytokinesis. Similar results were obtained when mutant cells were grown on a lawn of bacteria on a solid substratum. After depletion of the bacteria, cells developed and formed fruiting bodies.

DISCUSSION

The analysis of previous *Dictyostelium* mutants genetically engineered to eliminate conventional myosin expression demonstrated the essential role of this molecule in cytokinesis and development (1, 2). In addition, *Dictyostelium hmm* cells, which contain a truncated 140-kDa myosin and have an identical phenotype to myosin null cells, indicated the importance of an intact myosin molecule for its biological function (4). This raised the possibility that all major deletions in the myosin molecule might render the molecule dysfunctional in the cellular environment, which would result in the phenotype of the null cell. Here we report the creation of a *Dictyostelium* cell in which the wild-type 240-kDa *mhcA* gene was replaced with a gene that codes for a 200-kDa myosin containing an abbreviated tail. The cells carrying this *myΔC34* mutation lack one feature of myosin function, cytokinesis, but retain another, completion of the developmental cycle.

The mutant myosin encoded by HS2218 cells lacks the 34-kDa carboxyl terminus of wild-type 240-kDa myosin. Thus, the failure of the mutant cells to divide in suspension cultures could be due to a requirement for the 34-kDa carboxyl terminus of myosin during cytokinesis. Alternatively, the concentration of truncated myosin present in cells, which is less than half that of normal myosin in wild-type cells, may be insufficient to function in cytokinesis. If the latter were true, it would be possible to rescue the defective phenotype by constructing cells that overexpress the truncated myosin. Preliminary results indicate that some features of cytokinesis can be recovered in cells that express higher concentrations of the truncated myosin (T. Egelhoff, S. Brown, and J.A.S., unpublished observations).

Cells that lack myosin or that contain a truncated 140-kDa myosin (*hmm*) cannot ordinarily complete the *Dictyostelium* developmental program and instead progress only to the early aggregation stage. In contrast, cells carrying the *myΔC34* mutation were able to develop upon starvation and form fruiting bodies. Cells germinated from spores still contained only the mutant truncated myosin. Thus, whereas the 140-kDa *hmm* myosin cannot contribute to myosin activity *in vivo*, the 200-kDa myosin is sufficient for cells to complete the *Dictyostelium* developmental cycle. These results can be interpreted in the light of several *in vitro* studies of the *Dictyostelium* myosin molecule. Expression in *Escherichia coli* of different myosin tail fragments revealed that an internal 34-kDa segment of the myosin tail displays assembly properties characteristic of myosin filaments (6). This segment is included in the tail of the *myΔC34* myosin but not in the tail of the 140-kDa myosin. In addition, a proteolytically derived 200-kDa myosin fragment analogous to the truncated mutant myosin has solubility properties expected for a myosin capable of filament formation (9). These results suggest that inclusion of the assembly domain in the longer tail of the *myΔC34* myosin has allowed this molecule to function within the cellular environment. Myosin filament formation may be critical for the necessary shape changes cells undergo as they form the pseudoplasmodia or the "first finger" that rises from the aggregated mass of cells.

Our results unequivocally show that the 34-kDa carboxyl-terminal portion of *Dictyostelium* myosin is not required for completion of the *Dictyostelium* developmental cycle. This result is surprising because this portion of the myosin molecule represents about 20% of the total length of the myosin tail and has been suggested to contribute regulatory functions to the molecule. Two threonine residues that reside within the 34-kDa carboxyl terminus serve as phosphorylation sites for a purified myosin heavy-chain kinase isolated from vegetative cells (8). A different kinase purified from developed cells also phosphorylates threonine residues and requires the 34-kDa carboxyl terminus of myosin for phosphorylation (6). Threonine phosphorylation of *Dictyostelium* myosin increases transiently in developed cells in response to chemoattractant and is known to inhibit filament assembly *in vitro* (10, 11, 18, 19). Our results suggest that phosphorylation of these threonine residues on the myosin heavy chain by these two kinases is not a requirement for the development of *Dictyostelium* cells.

The tail portion of the *myΔC34* myosin contains serine residues that have been shown to be phosphorylated *in vivo* within vegetative cells (20). However, little is known about how serine phosphorylation affects the properties of myosin. Thus, it is not clear how inclusion of serine phosphorylation sites might permit myosin function within the cells.

The experiments reported here raise the question of the significance of threonine phosphorylation of the myosin heavy chain during development. A rapid and transient increase in threonine phosphorylation occurs early during development as wild-type cells migrate toward an aggrega-

tion center. Mutant cells which lack the threonine phosphorylation domain of myosin can accomplish this aspect of motility (Fig. 4*A*). However, while our results establish the nonessential nature of the carboxyl terminus, they do not demonstrate that the carboxyl terminus of myosin is insignificant or unimportant to cell physiology. Null cells and *hmm* cells can exhibit chemotaxis, but do so less efficiently than do wild-type cells. Analysis of the chemotactic behavior of *hmm* cells has shown that these mutant cells move at a reduced speed and are less polarized than wild-type cells (21). Thus, some contributions of myosin to cellular function can be subtle. Experiments such as these have led to the proposal that myosin functions as a modulator of cell shape and cell polarity in chemotactic cells (22). The broken streams of developing cells during chemotaxis, the lower number of migrating slugs, and the increased duration of the developmental cycle suggest that while HS2218 cells can complete the developmental cycle, they may not be as efficient as wild-type cells. This myosin mutant is unique in its ability to progress through stages of development for which previous myosin mutants were blocked. Video microscopy analysis of these cells throughout development will be important to understand the contribution of myosin to later stages of the developmental cycle.

Creation of the *myΔC34* mutant demonstrates the feasibility of experimental mutants that test the contribution of specific portions of myosin to *in vivo* functions. Further characterization of this mutant myosin and other engineered mutations will tell us more about how specific domains of the myosin tail contribute to biological function and how myosin contributes to cellular function.

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