Single-headed myosin II acts as a dominant negative mutation in Dictyostelium
(cytokinesis/capping)

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ABSTRACT Conventional myosin II is an essential protein for cytokinesis, capping of cell surface receptors, and development of Dictyostelium cells. Myosin II also plays an important role in the polarization and movement of cells. All conventional myosins are double-headed molecules but the significance of this structure is not understood since single-headed myosin II can produce movement and force in vitro. We found that expression of the tail portion of myosin II in Dictyostelium led to the formation of single-headed myosin II in vivo. The resultant cells contain an approximately equal ratio of double- and single-headed myosin II molecules. Surprisingly, these cells were completely blocked in cytokinesis and capping of concanavalin A receptors although development into fruiting bodies was not impaired. We found that this phenotype is not due to defects in myosin light chain phosphorylation. These results show that single-headed myosin II cannot function properly in vivo and that it acts as a dominant negative mutation for myosin II function. These results suggest the possibility that cooperativity of myosin II heads is critical for force production in vivo.

Our initial goal in the experiments described here was to test the importance of the density of myosin heads along a myosin thick filament, by overexpressing the tail segment (rod) of myosin II. Myosin rods and myosin can readily coassemble into the same thick filament, and the hybrid filaments can produce force in vitro (3). In addition to rods, the expressed tail peptide could form hybrid dimers with the endogenous MHC peptide forming single-headed myosin II. This molecule would contain a normal coiled-coil tail with a single myosin head. Both rods and single-headed myosin II should reduce the density of myosin heads on thick filaments. Surprisingly, we found that the expressed tail peptide assembled primarily into hybrid single-headed myosin II. The phenotype of the cells containing this hybrid molecule indicates that the two heads of myosin are indeed required for proper function in vivo.

MATERIALS AND METHODS

Cell Culture Growth, Transformation, and Test for Growth in Suspension. Conditions for growth of cultures and transformation of D. discoideum by electroperoration have been described (12). Clonal transformants were obtained by selection in HL5 medium containing G418 (10 μg/ml). All cell lines were maintained on Petri dishes in the same medium. To test for their ability to grow in suspension, each cell line was placed in an Erlenmeyer flask on an orbital shaker at 240 rpm and the titer was monitored for several days.

Design of Expression Vectors. To express myosin tail fragments in Dictyostelium, we used the plasmid pAD80HA, which is a modified version of plasmid pBS18 (kindly provided by Richard Firtel, University of California, San Diego). This vector contains a G418-selectable marker under the control of the actin-6 promoter and the actin-15 terminator. We modified this vector to express epitope-tagged proteins under the control of the actin-15 promoter and the SP70 terminator. This vector, pAD80HA, encodes an initiation methionine followed by the sequence recognized by the monoclonal antibody 12CA5 (YPYDVPDYA). The epitope tag is followed by restriction sites for Nco I, Kpn I, EcoRI, Bgl II, HindIII, and Kpn I. These sites were incorporated for the cloning and expression of specific MHC fragments.

Plasmid pAD80HA-ROD was obtained by cloning a 4.2-kb Nco I-Sma I fragment from pBlG4.5 (13) into the Nco I–EcoRI site of pAD80HA. This plasmid contains the entire myosin tail coding region (aa 809–2116, Fig. 1 and 260 bp of 3′ noncoding region. The predicted molecular mass of this fragment is 152 kDa. To obtain plasmid pAD80HA-S2, we simply removed from pAD80HA-ROD a 2.4-kb Kpn I fragment encompassing the light meromyosin portion and 3′ noncoding region. Therefore, plasmid pAD80HA-S2 encodes a myosin tail peptide from aa 809 to aa 1529 of the MHC

Abbreviations: MHC, myosin heavy chain; RMLC, regulatory myosin light chain.
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The tail peptide portion of the myosin II molecule (ROD) includes the assembly domain (necessary for filament formation) and the regulatory domain for myosin assembly. The S2 portion is defined here as the region of the myosin II molecule between the head and the assembly domain. The S2 portion is, therefore, unable to form filaments.

sequence. The predicted molecular mass of this fragment is 83.2 kDa.

**Western Blot Analysis.** The expression of myosin fragments was monitored by Western blot analysis (see below). The blot was incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody and developed by color reaction with 4-chloronaphthol and H₂O₂.

We used polyclonal antibody 9558-2 directed against the S2 portion of the *Dictyostelium* myosin II (D. Shoffner and A.D.L., unpublished results). This antibody recognizes both the expressed fragments and the endogenous MHC. We attempted to use the monoclonal antibody 12CA5 to detect specifically the expressed fragments since the vector incorporates this epitope tag but were unable to detect the two expressed fragments with this antibody. The absence of this epitope is not due to a mutation caused during construction of the vectors since we confirmed their sequence prior to transformation. Furthermore, the same vector has been used to epitope tag other proteins such as the light meromyosin (14). Therefore, the failure to detect the hemagglutinin epitope at the N terminus of the S2 and tail peptides could be due to proteolysis or inaccessibility in the context of their particular N-terminal sequence.

**Native Gel Electrophoresis.** A 20 mM sodium pyrophosphate/3% polyacrylamide gel (pH 8.5) was used to separate the different myosin species as described (3). Wild-type or mutant cells (2 × 10⁶ cells) were lysed in 1 ml of lysis buffer (0.1 M Mes, pH 6.8/0.5% Triton X-100/2.5 mM EGTA/1 mM MgCl₂/0.5 mM phenylmethylsulfonl fluoride), and their cytoskeletons were pelleted by microcentrifugation for 10 min at 4°C. The cytoskeletal pellets were resuspended in 0.06 ml of resuspension buffer (20 mM sodium pyrophosphate, pH 8.5/1 mM EDTA/0.01% 2-mercaptoethanol/300 mM NaCl/1 mM ATP). Samples were diluted 1:1 with loading buffer (20 mM sodium pyrophosphate, pH 8.5/1 mM EDTA/0.01% 2-mercaptoethanol/300 mM NaCl/10% (vol/vol) glycerol/0.006% bromophenol blue) and loaded onto the native PAGE gel.

Pure rod was isolated by first obtaining a cytoskeletal pellet as described above and then boiling the resuspended pellets. Myosin rod being a coiled-coil protein survives this treatment and remains soluble whereas all other proteins denature and sediment during microcentrifugation. The boiled extracts were microcentrifuged for 10 min at 4°C and the supernatants were loaded onto the native gel. The proteins in the native gel were transferred to nitrocellulose and probed with an anti-myosin antiserum as described above.

**Fractionation of Double- and Single-Headed Myosin.** Cytoskeletal pellets from wild-type and mutant cells were prepared as described above. The pellets were resuspended in 20 mM Tris·HCl, pH 8.0/0.5 M NaCl/1 mM ATP/1 mM MgCl₂/20 mM sodium pyrophosphate and centrifuged at 15,000 rpm in a SS-34 rotor for 30 min at 4°C. The supernatant containing myosin (0.5 ml) was loaded on a Superose-6 10/30 column. Myosin was eluted in an ~3-ml peak shortly behind the void volume. There was no separation of the different myosin species across this peak. The pooled fractions were concentrated to 0.5 ml on a Centricon-30, and 0.2 ml was loaded on a 5-ml gradient of 15–40% glycerol in 0.5 M ammonium bicarbonate. Gradients were centrifuged in the Beckman SW 50.1 rotor at 50,000 rpm, 20°C for 15 hr; 0.2-ml fractions were collected and analyzed by SDS/PAGE on Coomassie blue-stained gels. Comparison of the peak fractions to standard proteins sedimented in a separate gradient indicated approximate sedimentation coefficients of 5.3 S, 4.4 S, and 3.1 S for fractions 7, 9, and 11, respectively.

**Light-Chain Phosphorylation Assay.** Cells (3 ml at 1 × 10⁶ cells/ml) were pelleted onto duplicate 60-mm tissue culture plates and allowed to attach. Cells were then washed with Mes wash buffer (20 mM Mes/0.2 mM CaCl₂/2 mM MgSO₄, pH 6.8) and kept in 2.5 ml of the same buffer. Con A type IV (Sigma) was added to one of the plates, at 30 μg/ml, and incubated for 10 min. The cells were then released from the plates by pipetting up and down, and 1 ml was transferred to a tube containing 100 μl of 100% (wt/vol) trichloroacetic acid. The precipitated proteins were solubilized with 25 μl of 2× urea gel sample buffer, separated on a urea gel (15), and examined by Western blot analysis using a monoclonal antibody directed against the regulatory myosin light chain (RMLC) (antibody 1A2, kindly provided by Rex Chisholm, Northwestern University).

**RESULTS**

**Expression of Myosin Tail Fragments in Dictyostelium.** We designed *Dictyostelium* expression vectors for the production of tail segments of myosin II (Fig. 1). One vector, pAD80HA-ROD, encodes the entire myosin tail portion of the *Dictyostelium mhcA* gene. This portion of the MHC contains the assembly and regulatory domains that mediate the proper assembly of myosin filaments in the cell (16, 17). The expressed tail peptide should be able to form myosin rods or single-headed myosin II and incorporate into myosin filaments. As a control, we designed a vector, pAD80HA-S2, for the expression of myosin S2 fragments. This myosin fragment lacks the assembly domain and, therefore, would not incorporate into myosin filaments.

Both vectors were introduced into *Dictyostelium* AX3 cells. Clonal cell lines were isolated (ROD cells and S2 cells) that expressed the appropriate myosin fragments (Fig. 2). We determined by scanning gel densitometry that both fragments were expressed at levels close to those of the endogenous MHC (data not shown). Furthermore, the expression of either

**FIG. 1.** Myosin II tail fragments expressed in *Dictyostelium*. The tail peptide portion of the myosin II molecule (ROD) includes the assembly domain (necessary for filament formation) and the regulatory domain for myosin assembly. The S2 portion is defined here as the region of the myosin II molecule between the head and the assembly domain. The S2 portion is, therefore, unable to form filaments.

**FIG. 2.** Expression of myosin II tail fragments in *Dictyostelium*. The expression vectors were introduced into *Dictyostelium* wild-type AX3 cells. Western blot analysis of the isolated transformants probed with an anti-myosin II antiserum shows that the expected proteins are being expressed at levels similar to the endogenous MHCs. These blots also show that the expression of the endogenous MHC is not altered by the expression of the mutant fragments.
Expression of the Myosin Tail Peptide Disrupts Myosin II Function. We tested the two transformed cell lines for their ability to carry out cellular processes mediated by myosin II: cytokinesis, capping of cell surface receptors, and development (7, 9). We assayed for cytokinesis by placing each cell line in suspension cultures and monitoring their growth. We found that the wild-type and S2 cells grew at similar rates, whereas the ROD cells failed to grow under the same conditions (data not shown). This failure of the ROD cells to grow resulted from a deficiency in cytokinesis. These cells displayed a remarkable accumulation of nuclei and became very large in suspension conditions (Fig. 3A) whereas neither the wild-type cells (Fig. 3A) nor the S2 cells (data not shown) became large or multinucleate. When the same cell lines were treated with Con A, the wild-type (Fig. 3C) and S2 cells (data not shown) concentrated the bound ligand into a polar cap after a few minutes. However, even 30 min after treatment, the ROD cells did not produce caps (Fig. 3D). These experiments suggest that the expression of the myosin tail peptide interferes with normal myosin II function.

The ROD cells did retain some myosin II function. When placed in starvation conditions, the ROD cells completed the Dictyostelium developmental cycle and formed fully differentiated fruiting bodies in a time frame similar to that of wild-type and S2 cells (data not shown). Upon close inspection, however, we noticed that the fruiting bodies formed by the ROD cells were slightly smaller and more numerous than those from wild-type and S2 cells (data not shown). Therefore, the disruption of myosin II function by the myosin tail peptide seems to be most potent during cytokinesis and capping.

These experiments demonstrate that, despite having a full complement of MHC, the ROD cells are deficient in two important myosin II functions. One possible explanation is that the tail peptide causes the redistribution of myosin II to an inappropriate intracellular location. However, both indirect immunofluorescence microscopy of ROD cells stained with an anti-myosin antibody and analysis of the ROD cells by thin-section electron microscopy gave results that were indistinguishable from wild-type cells (data not shown).

![Fig. 3: Expression of myosin II rod disrupts cytokinesis and capping of Con A receptors](image1)

**Fig. 3.** Expression of myosin II rod disrupts cytokinesis and capping of Con A receptors. (A and B) Cells grown in suspension cultures for 2 days were fixed and stained with 4',6-diamidino-2-phenylindole (9). Wild-type cells (A) and S2 cells (data not shown) are mostly uninucleate cells. In contrast, the ROD cells (B) become large multinucleate cells indicating a failure in cytokinesis. (C and D) Wild-type and transformant cells were treated with Con A (9). Wild-type cells (C) and S2 cells (data not shown) cluster the bound Con A into polar caps within 5 min, whereas the ROD cells (D) are unable to do so even after 30 min. (Bars: A and B, 50 μm; C and D, 10 μm.)

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and by SDS/PAGE. Double-headed myosin II from wild-type cells was resolved as a single discrete peak in this gradient (Fig. 5A, fraction 7). In contrast, myosin II from the ROD cells was spread over several fractions. The heaviest fractions sedimented in the same position as the wild-type double-headed myosin II and contained only the full-length MHC (Fig. 5B, fractions 6 and 7). The intermediate fractions contained both MHC and myosin tail peptide in approximately equal amounts (Fig. 5B, fractions 8 and 9), a ratio consistent with a single-headed myosin II. Finally, the lightest fractions contained only the tail peptide (Fig. 5B, fractions 10 and 11) and, therefore, correspond to myosin rods without heads.

To confirm the identity of the different myosin II species, we analyzed the proteins from these fractions by rotary-shadowing electron microscopy. We found that fractions 7 and 10 contained predominantly double-headed myosin and myosin rod, respectively (Fig. 6). The intermediate fraction 9 contained a few double-headed myosins and rods, but the majority of myosin molecules (>80%) appeared to be single-headed. Therefore, based on the results of native gel electrophoresis, gradient sedimentation, and the identification of molecules in the electron microscope, we conclude that double- and single-headed myosin II and rods with no heads are present in the mutant cells.

Our results indicate that the expression of myosin tail peptide in Dictyostelium cells leads to the formation of single-headed myosin II. From densitometric scans of Western blots of native gels, we calculated an approximately equal ratio of double- and single-headed myosin II and only trace amounts of rod molecules without heads (data not shown). To estimate the amount of rod molecules in the mutant cells, we calculated the yield of purified rod from the boiled extracts. These estimates indicated that the amount of rod molecules is less than 10% of the amount of single-headed myosin II. The actual amount of rod molecules may be even smaller since rods can be easily produced in the cell extracts by proteolysis of single-headed myosin II. We have observed, for example, that the myosin preparations from ROD cells (Fig. 5B) are more susceptible to proteolysis than those from wild-type cells (Fig. 5A). Therefore, we think it is likely that the phenotypic defects observed in the ROD cells are a direct result of the presence of single-headed myosin II in these cells. Consequently, single-headed myosin II acts as a dominant negative mutation for myosin function.

Phosphorylation of the RMLC Is Normal in the ROD Mutant. One possible consequence of the presence of single-headed myosin in the ROD mutants is the disruption of myosin light chain phosphorylation. If phosphorylation of the RMLC is a cooperative event, then the lack of one head may disturb this process. To determine whether this type of cooperativity is responsible for the phenotype of the ROD cells, we analyzed the phosphorylation of the RMLCs in these cells. Dictyostelium wild-type cells phosphorylate the RMLC by a specific RMLC kinase in response to treatment with Con A (J. L. Smith, L. A. Silveira, and J. A. Spudich, personal communication) (Fig. 7, lanes 1 and 2). When the ROD mutant cells were treated with Con A, the RMLC was phosphorylated normally (Fig. 7, lanes 3 and 4). Thus, the regulation of myosin II by phosphorylation of the RMLC is not affected by the presence of single-headed myosin II.

**DISCUSSION**

We have shown herein that the in vivo expression of the myosin tail peptide in a wild-type background leads to the formation of single-headed myosin II and causes the loss of myosin function in Dictyostelium cells. This dominant negative effect is specific for the tail peptide since the expression of the myosin S2 peptide produces no detectable consequences. We find that most of the expressed tail peptide is associated with a full-length MHC peptide forming single-headed myosin II molecules. Therefore, the dominant negative effect observed in these mutant cells is associated with the presence of single-headed myosin II.

Although nothing is known about the mechanisms of coiled-coil formation in vivo, it is likely that the dimerization of two MHC peptides occurs post-translationally. This hypothesis is supported by the presence of myosin heterodimeric molecules in rat cardiac tissue and smooth muscle (19, 20). Our results also support this hypothesis since we find heterodimeric single-headed myosin II molecules. However, it is not clear why the ROD mutant cells contain very few rod peptide homodimers. We do not think that this bias from a random distribution is caused by instability of the rod molecules within the cell. Myosin rods have been expressed in a Dictyostelium myosin null cell line without any obvious signs of instability (T. Egelhoff and J. A. Spudich, personal communication). One possible explanation for the observed distribution is that the tail peptide forms coiled-coils at a slower rate than the MHC peptide. This could be caused by the unnatural N terminus of the tail peptide or the few extra amino acids added by the expression vector. In this situation, the MHC peptide may drive most of the tail peptides to form heterodimeric coiled-coils. Only a few tail peptides would self-associate to form rods. This phenomenon would not occur in a myosin-null cell, where all the tail peptides would have ample time to self-associate.

In contrast to cytokinesis and capping, the expression of myosin tail peptide did not affect significantly the developmental cycle of the ROD mutant cells. The only appreciable effect was the formation of slightly smaller and more numerous fruited bodies of ROD cells compared to wild-type cells. It is likely that the lack of effect on development results from the loss of single-headed myosin molecules in developing cells. This loss would be caused by a change in the levels of expression of the MHC and myosin tail peptide genes during development. While the MHC gene expression levels increase during early development (21), those from the actin-15 promoter used in the expression vector drop sharply after 6 hr of development.

**FIG. 6.** Rotary-shadowing electron microscopy of the purified myosin species. Fractions from the glycerol gradient of ROD cells (Fig. 5) were sprayed onto mica and rotary shadowed (18). Molecules representative of the majority species in the three fractions are shown. A similar preparation of myosin from wild-type cells gave only double-headed myosin (data not shown). (Bar = 100 nm.)
development (22). Thus the formation of single-headed myosin molecules would cease soon after development is initiated.

Why is a single-headed myosin so detrimental for myosin function in vivo when it displays apparently normal function in vitro (3)? A trivial explanation would be that the single-headed molecules are completely inactive and, as a consequence, the cell has a reduced level of active myosin molecules. Since the ROD mutants contain about an equal ratio of single- and double-headed myosin II, this explanation would predict a level of at least 50% of active myosin. This explanation seems unlikely in the light of many other experiments with myosin II mutants; those mutants that express as little as 42% of normal levels are still able to undergo cytokinesis and capping (23). Another possibility is that the single-headed myosin II molecules bind to the actin filaments but are not able to produce force. This unproductive binding would generate a drag force on the sliding movement of actin and myosin filaments and may disrupt force production in vivo. We believe this possibility is unlikely because we did not observe a rigor binding of the single-headed myosin molecules to the actin cytoskeletons during our purification experiments. However, a direct measurement of the in vitro ATPase and movement rates of the purified single-headed molecules will be necessary to address this possibility.

A more attractive possibility suggested by our results is that an important cooperative aspect of myosin II function has been affected in vivo. In smooth muscle, for example, the activation of myosin II by light chain phosphorylation is a cooperative phenomenon along a myosin filament (24). It is possible that the phosphorylation of the RMLC in the single-headed myosin may not be as efficient as in a double-headed molecule. Furthermore, the incorporation of single-headed myosin into filaments may disturb the phosphorylation of the double-headed molecules in the same filament. We believe this is an unlikely possibility since we have found that phosphorylation of the RMLC in the ROD mutant cells is normal in response to stimulation with Con A. Another cooperative function that could be perturbed in the mutant cells is the cooperative contribution of myosin heads to force production. It is known that single-headed myosin II binds to actin much more weakly than double-headed myosin under certain conditions (25). Although the absence of one head does not detract from motility when myosins are bound randomly to glass, the cooperative or sequential binding of two heads may play a crucial role for motility along a myosin filament in vivo. It is possible that myosin heads need to be precisely distributed along the surface of the filament to achieve force production in vivo. The absence of some heads along the filament may, therefore, disturb the entire process.

Finally, it is possible that the single-headed myosin is no longer regulated by phosphorylation in vivo. Recent studies have shown that single-headed myosin from smooth muscle is fully active independently of the phosphorylation state of the remaining RMLC (26). If myosin function in vivo requires the ability to "turn off" myosin molecules by dephosphorylation, then the presence of unregulated single-headed molecules may severely disrupt myosin function in the cell. This hypothesis may also explain the results obtained with a mutation in the phosphorylation site of the *Dictyostelium* RMLC (15). When Ser-13 was replaced by Ala, the resultant myosin displayed only a basal ATPase activity similar to unphosphorylated *Dictyostelium* myosin. Surprisingly, cells carrying this mutation have a completely normal phenotype, indicating that activation of myosin II by phosphorylation is not essential for myosin function in vivo. However, if the important aspect for myosin function is to reduce its activity by dephosphorylation, then the mutation described above would be predicted to have no major effect in vivo. On the other hand, a Ser→Asp change may mimic the phosphorylated RMLC and result in a constitutively active myosin molecule. Our results would suggest that this mutation may have a dominant negative phenotype.

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