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Involvement of Tail Domains in Regulation of *Dictyostelium* Myosin II

Xiong Liu,*,[1] Kohji Ito,*,[2] Randall J. Lee,† and Taro Q. P. Uyeda*

**Biomolecular Research Group, National Institute for Advanced Interdisciplinary Research, Tsukuba, Ibaraki 305-8562, Japan; and †Department of Medicine and Cardiovascular Research Institute, University of California at San Francisco, San Francisco, California 94134*

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The actin-dependent ATPase activity of *Dictyostelium* myosin II filaments is regulated by phosphorylation of the regulatory light chain. Four deletion mutant myosins which lack different parts of subfragment 2 (S2) showed phosphorylation-independent elevations in their activities. Phosphorylation-independent elevation in the activity was also achieved by a double point mutation to replace conserved Glu932 and Glu933 in S2 with Lys. These results suggested that inhibitory interactions involving the head and S2 are required for efficient regulation. Regulation of wild-type myosin was not affected by copolymerization with a S2 deletion mutant myosin in the same filaments. Furthermore, the activity linearly correlated with the fraction of phosphorylated molecules in wild-type filaments. These latter two results suggest that the inhibitory head-tail interactions are primarily intramolecular.

Key Words: **myosin; regulatory light chain; regulation; phosphorylation; *Dictyostelium*.**

The activity of conventional myosin or myosin II (referred to as myosin hereafter) from smooth muscle and a number of nonmuscle cells is regulated by phosphorylation of the regulatory light chain (RLC) (1). While the RLC is certainly a key element in the regulation, the myosin tail region is also essential for regulation. Firstly, smooth muscle myosin subfragment 1 (S1), which is single-headed and lacks the tail region, is always in the ON state regardless of the phosphorylation state (2, 3), whereas heavy meromyosin (HMM), which consists of two heads and a certain length of the truncated tail, is well regulated by phosphorylation of the RLC (4). Recent experiments using truncated molecules further demonstrated that the interaction between the two RLC domains on the two heads and/or a motor domain and a RLC domain in a distinct head is required for the inhibition of smooth muscle myosin activity when RLC is dephosphorylated (5). These data suggest that the primary role of the tail region in smooth muscle regulation is to hold the two motor domains in close proximity. However, Trybus *et al.* (6) showed that dimerization by the tail domain is not sufficient and a certain length of the tail is required for efficient regulation, suggesting the possibility that an interaction between the head and the tail is also necessary. Thus, functions of the tail domain in smooth muscle regulation is not fully understood.

Myosin of the cellular slime mold *Dictyostelium discoideum* is also positively regulated by phosphorylation of RLC (7–10). Unlike smooth muscle myosin, however, a global conformational change associated with RLC phosphorylation has not been observed so far (1, 7, 11) and filament assembly is not affected by RLC phosphorylation (7). Through analyses of a number of myosins carrying mutations in the distal tail region, we recently found that HMM is not efficiently regulated and a filament structure is essential for efficient regulation by RLC phosphorylation in *Dictyostelium* myosin (12). This is another indication that the regulatory mechanism of *Dictyostelium* myosin is rather different from that of smooth muscle myosin. The results reported here further shows that the proximal tail domains are also required for efficient regulation of *Dictyostelium* myosin by RLC phosphorylation. Possible roles of the proximal tail region in the regulation are discussed.

MATERIALS AND METHODS

Generation of mutant myosins. Standard methods were used for all DNA manipulations (13).

MyΔ943–1464 was reported previously (14), except that it was incorrectly identified as MyΔ934–1454. Construction of MyΔ824–941, MyΔ943–1194, and MyΔ1157–1464 were described elsewhere (15).

[1] Present address: Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20892.

[2] Present address: Department of Biology, Faculty of Science, Chiba University, Inage, Chiba 263-8522, Japan.

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The point mutations were made on pMyDAP (16) using a Pharmacia USE site-directed mutagenesis kit. The sequences of the mutagenic primers were: 5′GAAAAAGTCAGAGATTTAAAAAAAGAATTACAAGAG for MyEE/KK, 5′CCGTGTTGAAGATATGAAATCTAAACTAGACGAAAAG for My E904E906/KK and 5′GGAATTAGAAGAAATGGAAGAAGTCAATGACGG for MyK960R961/EE.

The fragment of each mutant gene was employed to replace the corresponding wild-type sequence in pTIKLMyDAP using appropriate restriction sites. The sequences of these DNA fragments were confirmed by dideoxy sequencing. pTIKL is the same as pBIG (17), except that the two *Nco*I sites in the neomycin resistance cassette are removed by site-directed mutagenesis, and the ~1 kb *Kpn*I (blunt)-*Kpn*I (blunt) fragment is removed from the DdpI extrachromosomal replication sequence. The *Xba*I-*Sac*I fragment of pMyDAP was subcloned at the corresponding sites of pTIKL to yield pTIKLMyDAP.

E476KΔ824–941 was constructed by subcloning *Eco*NI-*Bgl*II fragment of pBIGMyDE476K (18) into pTIKLMyDΔ824–941.

Construction of a chimeric gene coding for green fluorescent protein (GFP) fused to the N-terminus of wild-type myosin heavy chain was reported previously (19). After introduction of a S65T mutation in the GFP moiety, the chimeric gene was subcloned into pTIKL (20).

The resultant pTIKLMyDAP carrying each mutation was transformed into *Dictyostelium* cells that lack the endogenous copy of *mhc*A (17) by electroporation (21) and transformants were selected in the presence of 12 μg/ml G418 in the HL5 medium containing 60 μg/ml each of penicillin and streptomycin (PS).

Culture of Dictyostelium cells. All transformants were maintained on plastic plates containing HL5 + PS + 12 μg/ml G418 at 13°C. For large scale cultures, wild-type Ax2, MyEE/KK, MyE904E906/KK and MyK960R961/EE cells were grown in 2 l of HL5 + PS without G418 in a 5-l flask on a rotary shaker at 23°C. Cells expressing MyΔ824–941, MyΔ943–1157, MyΔ1194–1464, MyΔ943–1464 and MyE476KΔ824–941, which did not grow well in suspension, were grown in HL5 + PS and 6 μg/ml G418 on 25 × 25 cm square plastic plates at 23°C.

Preparation of proteins. All procedures were carried out at 4°C. *Dictyostelium* myosins were purified by the method of Ruppel *et al.* (17) with some modifications. Cells (10–25 g) were washed with 10 mM Tris (pH 7.5) and resuspended in 5 vol/g cell of a lysis buffer (25 mM Hepes, 2 mM EDTA, 1 mM DTT and 50 mM NaCl, pH 7.4). Lysis buffer (4 vol/g cells) containing Triton X-100 was added, and the suspension was gently mixed. The final concentration of Triton X-100 was 0.4% for wild-type and 0.6% for mutant cells. The lysate was immediately centrifuged at 36,000*g* for 15 min. The supernatant was removed and the pellet was centrifuged again for 5 min to remove remaining supernatant. The pellet was homogenized in 9 vol/g cells of a washing buffer (10 mM Hepes, 150 mM NaCl, 3 mM $MgCl_2$ and 1 mM DTT, pH 7.4), and the homogenate was immediately centrifuged at 36,000*g* for 20 min. The final pellet was homogenized in 1.5 vol/g cells of an extraction buffer (10 mM Hepes, 125 mM NaCl and 1 mM DTT, pH 7.4) and ATP was added to 5 mM. The supernatant after centrifugation at 200,000*g* for 30 min was supplemented with preboiled RNase A to 5 μg/ml and was dialyzed against a buffer containing 10 mM Pipes, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT (pH 6.8) for >8 h. The precipitates were recovered by centrifugation at 36,000 g for 15 min and were resuspended in 0.2–0.5 vol/g cells of a buffer containing 10 mM Hepes, 300 mM NaCl, 1 mM DTT and 0.5 mM ATP (pH 7.4). After supplemented with additional ATP to 3 mM, the solution was centrifuged immediately at 220,000*g* for 30 min. The supernatant was diluted 5-fold with a buffer containing 10 mM Pipes, 10 mM $MgCl_2$ and 1 mM DTT (pH 6.8) and left on ice for 40 min. The assembled myosin was recovered by centrifugation at 110,000*g* for 12 min and were dissolved in 0.05 vol/g cells of a buffer containing 10 mM Hepes, 250 mM NaCl, 2 mM ATP and 1 mM DTT (pH 7.4). The solution was clarified by centrifuging at 220,000*g* for 10 min. The lysis, washing and extraction buffers contained 0.1 mM phenylmethylsulfonylfluoride, 2 μg/ml pepstatin, 5 μg/ml leupeptin, 80 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone, 50 μg/ml Na-p-tosyl-L-lysine chloromethyl ketone and 5 mM benzamidine.

Rabbit skeletal muscle actin and myosin were prepared using the method of Spudich and Watt (22) and Margossian and Lowey (23), respectively. The concentrations of these proteins and purified *Dictyostelium* myosin were determined as described previously (12).

Phosphorylation of *Dictyostelium* myosin was performed as described previously (12).

ATPase assays. Steady state ATPase activities were determined by measuring release of phosphate as described previously (12). The reaction mixtures for the assay of MgATPase activity contained 10 mM imidazole (pH 7.5), 25 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, and 0.1 mg/ml myosin, with or without 1 mg/ml F-actin. Reactions were started by the addition of myosin.

Electrophoretic methods. SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the Laemmli system (24). Urea-SDS-glycerol polyacrylamide gel electrophoresis was performed as described previously (12).

Filament coassembly assays. Solutions of GFP-wild-type myosin fused protein and MyΔ934–1454 in buffer containing 10 mM Hepes, 250 mM NaCl, 2 mM ATP and 1 mM DTT (pH 7.4) were diluted, either individually or in a mixture, into a buffer containing 4 mM $MgCl_2$, 2 mM ATP, 10 mM Hepes (pH 7.4), 1 mM DTT, 0.2 mg/ml BSA, and variable concentrations of NaCl so that its final concentration after dilution would be 25 mM. The solutions were then centrifuged at 50,000 rpm for 15 min, and the supernatants and precipitates were analyzed by SDS–PAGE.

RESULTS

The schematic diagrams of the deletion mutations are shown in Fig. 1. Initial attempts to purify these mutant myosins using the method of Ruppel *et al.* (17) proved unsuccessful, resulting in severe degradation of RLC (result not shown). The modified protocol described under Materials and Methods is still simple, rapid and highly reproducible, and more significantly, degradation of RLC was not observed among mutant myosins purified using this improved method (Fig. 2).

Wild-type and the mutant myosins were recovered predominantly with dephosphorylated RLCs (Fig. 3). Treatment with bacterially expressed *Dictyostelium* myosin regulatory light chain kinase (25) almost fully phosphorylated the RLC of each purified myosin (Fig. 3). These unphosphorylated and phosphorylated myosins were subjected to ATPase assays (Table 1). The basal MgATPase activities of MyΔ824–941, MyΔ943–1194, MyΔ1157–1464 and MyΔ943–1464 in the absence of F-actin were much higher than that of wild type. The actin-dependent MgATPase activities of unphosphorylated mutant myosins were also much higher than that of unphosphorylated wild type and were even higher than that of phosphorylated wild type. The increase in actin-dependent ATPase activity of wild-type myosin by phosphorylation of RLC was approximately 6-fold, which is consistent with the published values (7, 17, 26). The increase of actin-dependent ATPase activities by RLC phosphorylation of MyΔ824–941, MyΔ943–1194, MyΔ1157–1464 and MyΔ943–1464 was 1.4-, 1.2-, 1.2-, and 1.3-fold (average

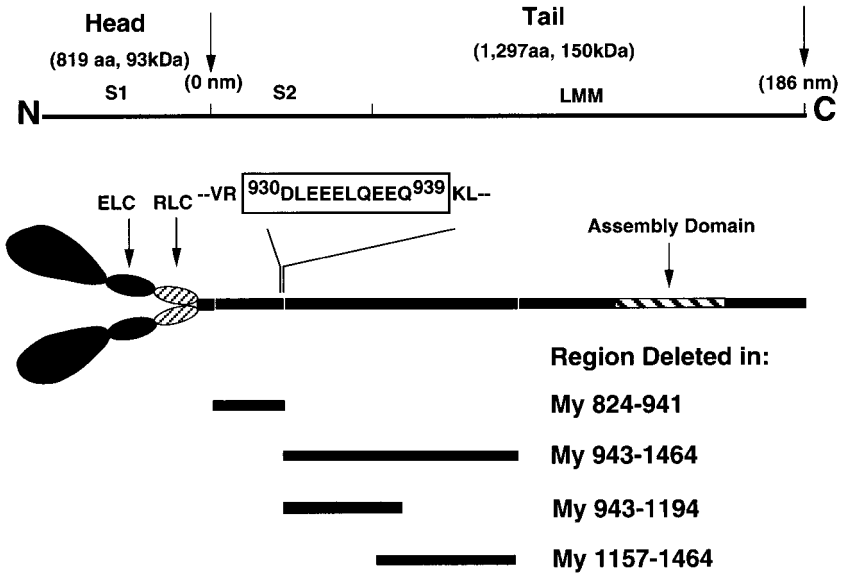

FIG. 1. Schematic representations of *Dictyostelium* myosin. The top line shows the relative scale of the tail region. The S2/LMM junction was defined as the region susceptible to chymotrypsin cleavage in *Dictyostelium* myosin. (34, 35). The boxed sequence starting at 110 amino acid residues from the head-tail junction is highly conserved among myosins that are regulated by RLC phosphorylation.

of the two independent experiments shown in Table 1), respectively. These data indicate that the inhibition by the dephosphorylated RLC was greatly relieved by deletion of some sequences in the S2 region. It was not clear, however, if the loss of regulation was simply due to shorter length of the tail or removal of some specific site(s). To test the latter possibility, we looked for a candidate specific sequence in the tail near the head-tail junction which may contribute to regulation.

A unique, negatively charged segment, ^{930}DLEEELQEEQ^{939}, starting at 114 amino acid residues downstream of the head-tail junction, was found only among myosins that are tightly regulated by RLC phosphorylation (Fig. 4). To test the possible involvement of these negative charges in regulation, we made a double point mutation, in which the first two Glu were replaced with Lys (MyEE/KK). The actin-dependent ATPase activity of unphosphorylated MyEE/KK was about 2-fold higher than the unphosphorylated wild-type, while that of phosphorylated MyEE/KK was almost the same as the phosphorylated wild-type myosin (Table 2). The EE/KK mutation halved the fold increase of actin-dependent ATPase activity by RLC phosphorylation. In order to examine the specificity of the effects of this mutation, we constructed two control double point mutant myosins, MyE904E906/KK and MyK960R961/EE. The mutation sites were 28 amino acid residues upstream and downstream of the EE/KK site respectively. Like the wild type, these two mutant myosins were well regulated by RLC phosphorylation (Table 2). These results suggest that the DLEEELQEEQ sequence is specifically involved in regulation by RLC

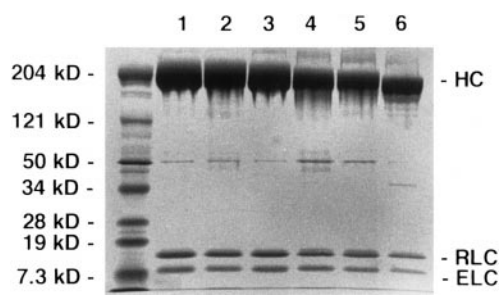

FIG. 2. SDS–PAGE analysis of *Dictyostelium* myosins used in this study. The upper half of separating the gel contained 7.5% acrylamide and the lower half contained 14% acrylamide and 5% glycerol. They were all electrophoretically homogeneous with no detectable degradation of the RLC. From lanes 1 to 6 are wild-type, MyEE/KK, MyΔ824–941, MyΔ943–1194, MyΔ1157–1464, and MyΔ943–1464.

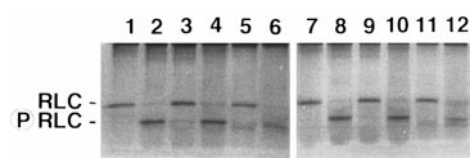

FIG. 3. Urea-SDS-glycerol polyacrylamide gel electrophoresis analysis of RLC phosphorylation of *Dictyostelium* myosin. RLC indicates dephosphorylated RLC, and P + RLC represents the phosphorylated form. From lanes 1 to 12 are control wild-type, kinase-treated wild-type, control MyEE/KK, kinase-treated MyEE/KK, control MyΔ824–941, kinase-treated MyΔ824–941, control MyΔ943–1194, kinase-treated MyΔ943–1194, control MyΔ1157–1464, kinase-treated MyΔ1157–1464, control MyΔ943–1464, and kinase-treated MyΔ943–1464. All six myosins were poorly phosphorylated before kinase treatment, and were almost fully phosphorylated after the treatment with kinase.

TABLE 1

MgATPase Activities of Truncation Mutant Myosins

Myosins	Actin	Kinase treatment −	Kinase treatment +	Fold increase by RLC phosphorylation
Wild-type (n = 3)	−	0.023 ± 0.008	0.032 ± 0.002	1.4
(n = 5)	+	0.13 ± 0.03	0.74 ± 0.10	6.0
MyΔ824–941	−	0.081	0.080	1.0
	−	0.082	0.072	0.9
	+	0.99	1.63	1.7
	+	1.39	1.71	1.2
MyΔ943–1194	−	0.048	0.066	1.4
	−	0.060	0.068	1.1
	+	1.56	1.71	1.1
	+	1.20	1.58	1.3
MyΔ1157–1464	−	0.078	0.068	0.9
	−	0.074	0.071	1.0
	+	1.32	1.81	1.4
	+	1.80	1.84	1.0
MyΔ943–1464	−	0.060	0.088	1.5
	−	0.088	0.085	1.0
	+	1.09	1.43	1.3
	+	1.38	1.72	1.3

Note. Activities are shown as Pi liberated per head per s. Results of mutant myosins are from two independent experiments. The data of wild-type myosin are compilation from three independent experiments.

phosphorylation, and that the tail sequences or structure somehow affect the activity of the motor domain.

This head-tail communication may be intramolecular or may depend on intermolecular interactions between neighbor molecules within filaments. To distinguish between these two possibilities, we examined regulation of copolymers consisting of wild type myosin and mutant myosin with an inactive head and a tail with the Δ824–941 deletion.

	0 109 122
Dictyostelium *	--P--VRDLEEELQEEQKL-
Fruit fly embryo*	--P--IQDLEEQLEEEEAA-
Chicken nonmuscle*	--P--IQELEEQLEEEESA-
Human nonmuscle*	--P--IQELEEQLEEEESA-
Rabbit smooth muscle*	--P--MLDLEEQLEEEEAA-
Chicken gizzard*	--P--MLDLEEQLEEEEAA-
Acanthamoeba	--P--KGELKASLEEEERN-
Scallop skeletal muscle	--P--NANLKKDIGDLENT-
Chicken Skeletal muscle	--P--CSELKKDIDDLELT-
Rat cardiac muscle	--P--CSELKKDIDDLELT-

FIG. 4. Comparison of amino acid sequences of the tail domain near the head-tail junction of myosins from a variety of sources. "P" denotes the proline residue which marks the junction between the head and the tail (36). The numbers in the top line are the distance from this proline residue. The species with * are tightly regulated by RLC phosphorylation. The underlined amino acid residues are conserved among these myosins. Protein sequences were obtained from Gene/Protein Sequence Database 5, Protein Identification Resource R42.0, 1994.

We first examined whether myosin molecules with intact tail and those with the Δ824–941 internal deletion coassemble, when they were mixed in a high salt buffer and then diluted to a low-salt buffer to promote filament assembly. For this experiment, wild-type myosin fused to GFP at the N-terminus was used in place of native wild-type myosin, so that the heavy chain of two myosins can be better resolved by SDS-PAGE. Under the conditioned used, both GFP-wild-type myosin and MyΔ824–941 at a concentration of 1.5 nM stayed in the supernatant after ultracentrifugation in a low salt buffer (Fig. 5, lanes 2 and 7). However, when 1.5 nM GFP-wild type myosin or MyΔ824–941 was mixed with 150 nM carrier myosin (MyΔ824–941 for GFP-wild type myosin, and GFP-wild type myosin for MyΔ824–941) in 250 mM NaCl and then diluted into a

TABLE 2

Actin-Dependent MgATPase Activities of Point Mutant Myosins

Myosins	Kinase treatment −	Kinase treatment +	Fold increase by RLC phosphorylation
Wild-type (n = 5)	0.13 ± 0.03	0.74 ± 0.10	6.0
MyEE/KK (n = 4)	0.30 ± 0.09	0.74 ± 0.02	2.9
MyE904E906/KK	0.14	0.90	6.4
MyK960R961/EE	0.12	0.68	5.9

Note. Activities are shown as Pi liberated per head per s.

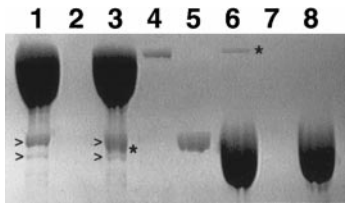

FIG. 5. Formation of copolymers between GFP-wild-type myosin and MyΔ824–941. Solutions of GFP-wild-type myosin and MyΔ824–941 in a high salt buffer were diluted to the low salt ATPase assay buffer, either individually or after mixing with each other as indicated below. Samples were then subjected to ultracentrifugation, and supernatant and pellet fractions were run on a 6% polyacrylamide gel. Pellet fraction of 150 nM (final concentration) GFP-wild-type myosin, lane 1; pellet fraction of a mixture of 150 nM GFP-wild type myosin and 1.5 nM MyΔ824–941, lane 3; supernatant fraction of a mixture of 150 nM GFP-wild-type myosin and 1.5 nM MyΔ824–941, lane 4; pellet fraction of 1.5 nM MyΔ824–941, lane 2; pellet fraction of 150 nM MyΔ824–941, lane 8; pellet fraction of a mixture of 1.5 nM GFP-wild-type myosin and 150 nM MyΔ824–941, lane 6; supernatant fraction of a mixture of 1.5 nM GFP-wild-type myosin and 150 nM MyΔ824–941, lane 5; pellet fraction of 1.5 nM GFP-wild-type myosin, lane 7. ">" indicates minor contaminating polypeptides contained in the GFP-wild-type myosin preparation. 1.5 nM GFP-wild-type myosin or MyΔ824–941 does not form pelletable polymers (lanes 7 and 2, respectively), but does so in the presence of 150 nM MyΔ824–941 or GFP-wild-type, respectively, as indicated by asterisks in lanes 3 and 6.

low salt buffer, both myosins precipitated efficiently (Fig. 5, lanes 3 and 6). These results indicate that wild-type myosin and MyΔ824–941 coassemble and form copolymers.

E476K mutation (18), which suppresses the actin-dependent ATPase activity to less than four hundredth of the wild-type activity (27), was used to make an enzymatically inactive head. Copolymers of wild type myosin and double mutant myosin with an inactive head and a tail with the internal deletion (MyE476KΔ824–941) were made, and the ATPase activities deriving from the wild type molecules were assayed. The specific activities of both phosphorylated and unphosphorylated wild-type and the extent of regulation were unaffected by the presence of varying fraction of MyE476KΔ824–941 in the copolymer (Fig. 6). This result strongly suggested that the inhibitory interaction between the tail and the catalytic domain is primarily intramolecular.

It was also shown that there existed a linear relationship between the activity and the fraction of phosphorylated molecules in wild-type homopolymers (Fig. 7). This result demonstrates that each myosin molecule is regulated individually, rather than regulated by the average phosphorylation level of each filament.

DISCUSSION

We have demonstrated that mutant *Dictyostelium* myosins which carry an internal deletion in the S2 region of the tail have increased actin-dependent MgATPase activities and are in the ON state irrespective of the RLC phosphorylation state (Table 1). Furthermore, a specific set of negatively charged residues in the S2 region is required for efficient regulation (Table 2). These results can be interpreted in the context of two different models: that is, the mutations may disturb direct, inhibitory association between the head and the tail, or the mutations may impact a specific filament structure required for efficient regulation.

The model that direct association between the two heads and the tail domain within each molecule are essential for regulation is based on the electron microscopic observation that the two heads bend back along the S2 region of monomeric smooth muscle myosin (28). Recent results that single-headed smooth muscle myosin consisting of one head and a dimerized tail is unregulated by RLC phosphorylation (5, 29, 30) suggest that the two heads interact with the tail in such a way that one head masks critical site(s) of the other. This model may be applicable to *Dictyostelium* myosin

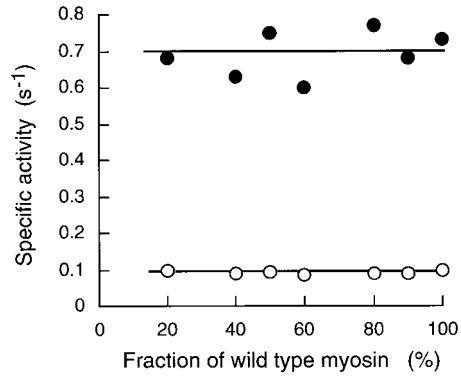

FIG. 6. Effect of adding MyE476KΔ824–941 to the wild-type myosin filaments. Phosphorylated (closed circles) or unphosphorylated form (open circles) wild-type myosin was mixed with MyE476KΔ824–941 at various ratios and the actin-dependent ATPase activities of copolymers were measured.

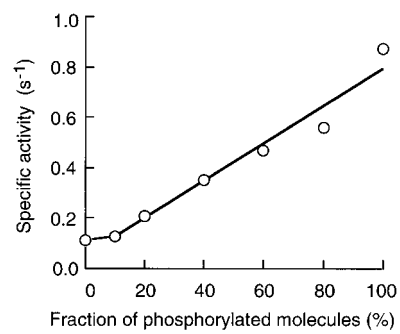

FIG. 7. Relationship between the actin-dependent ATPase activity and the fraction of phosphorylated molecules in wild-type myosin filaments. The various levels of RLC phosphorylation was obtained by mixing the fully phosphorylated form with the unphosphorylated form.

also, because single headed *Dictyostelium* myosin is unregulated as well (31). Negative charges in the ^{930}DLEEELQEEQ^{939} site, which are specifically required for the regulation of *Dictyostelium* myosin, may be involved in this head-tail interactions.

Past structural studies on *Dictyostelium* myosin do not support the model that direct head-tail association is involved in the regulation by RLC phosphorylation, however. Electron microscopic observation of unphosphorylated monomeric *Dictyostelium* myosin, which should be predominantly dephosphorylated (Fig. 3), failed to detect a bent structure around the head-tail junction even in the absence of salts (11, 14, 32). We recently showed that filament formation is required for efficient regulation of *Dictyostelium* myosin by phosphorylation of RLC, a situation that is quite different from smooth muscle myosin (12). Therefore, it is possible that the hypothetical inhibitory head-tail association in *Dictyostelium* myosin depends on filament assembly and hence was undetected in studies using monomeric molecules. Alternatively, regulation of *Dictyostelium* myosin by RLC phosphorylation may not depend on direct head-tail associations but rather involve changes of the filament structure induced by RLC phosphorylation. The activity of *Acanthamoeba* myosin is negatively regulated by phosphorylation at the distal end of the tail, when assembled into filaments (reviewed in 33). Thus, it is possible that a similar communication between heads and tails in filaments is involved in the regulation of *Dictyostelium* myosin by RLC phosphorylation.

The coiled-coil arrangement of 7 residue repeat units and the packing repeat units of 28 residues to form bipolar filaments are preserved in MyΔ943–1194 and MyΔ1157–1464 deletions. Furthermore, earlier electron microscopic observation of the larger deletion (MyΔ943–1464) did not reveal noticeable structural alteration of the filaments, except that these filaments were shorter than the wild-type filaments (14). The EE/KK point mutation, which does not disturb the 7 residue repeat, is also unlikely to impact the filament structure in general. Thus, these deletion and substitution mutations impair efficient regulation without disturbances to the global filament structure. Also we have recently shown (12) that the skeletal myosin tail cannot substitute for *Dictyostelium* myosin tail in terms of supporting efficient regulation. Even an in-frame substitution of the light meromyosin domain, which is distal to the S2 domain, with that of skeletal muscle myosin impaired efficient regulation. These results demonstrate that a highly specific filament structure is required for efficient regulation of *Dictyostelium* myosin, either by facilitating inhibitory head-tail associations or communications.

The tail appears to be involved in this regulatory process through intramolecular association or communications with the catalytic domain. This conclusion is based on the observation that doping of the wild-type myosin filaments with MyE476KΔ824–941, which has no enzyme activity but is in the ON state with respect to regulation, did not affect the regulation of wild-type (Fig. 6). Furthermore, the average activity of copolymer of phosphorylated and unphosphorylated wild type myosins was linearly related to the fraction of phosphorylated molecules (Fig. 7). This result suggests that the activity of each molecule within filaments is regulated individually and does not support the hypothesis that the global filament structure affects the activity of individual molecules.

Taken together, it is suggested that a specific filament structure is required to allow appropriate inhibitory interactions between the heads and the tail within each individual *Dictyostelium* myosin molecule. This is very different from inhibition of activities of dephosphorylated smooth muscle myosin, which does not depend on filament assembly or specific sequences in the tail. Structural studies to reveal phosphorylation-induced conformational changes around the head-tail junction in filaments are warranted.

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REFERENCES

1. Tan, J. L., Ravid, S., and Spudich, J. A. (1992) Control of nonmuscle myosins by phosphorylation. *Annu. Rev. Biochem.* **61,** 721–759.
2. Seidel, J. C. (1980) Fragmentation of gizzard myosin by alpha-chymotrypsin and papain, the effects on ATPase activity, and the interaction with actin. *J. Biol. Chem.* **255,** 4355–4361.
3. Ikebe, M., and Hartshorne, D. J. (1985) Proteolysis of smooth muscle myosin by Staphylococcus aureus protease: Preparation of heavy meromyosin and subfragment 1 with intact 20000-dalton light chains. *Biochemistry* **24,** 2380–2387.
4. Sellers, J. R. (1985) Mechanism of the phosphorylation-dependent regulation of smooth muscle heavy meromyosin. *J. Biol. Chem.* **260,** 15815–15819.
5. Li, X., Saito, J., Ikebe, R., Mabuchi, K., and Ikebe, M. (2000) The interaction between the regulatory light chain domains on two heads is critical for regulation of smooth muscle myosin. *Biochemistry* **39,** 2254–2260.
6. Trybus, K. M., Freyzon, Y., Faust, L. Z., and Sweeney, H. L. (1997) Spare the rod, spoil the regulation: necessity for a myosin rod. *Proc. Natl. Acad. Sci. USA* **94,** 48–52.
7. Griffith, L. M., Downs, S. M., and Spudich, J. A. (1987) Myosin light chain kinase and myosin light chain phosphatase from *Dictyostelium:* Effects of reversible phosphorylation on myosin structure and function. *J. Cell Biol.* **104,** 1309–1323.
8. Tan, J. L., and Spudich, J. A. (1990) Dictyostelium myosin light

chain kinase. Purification and characterization. *J. Biol. Chem.* **265,** 13818–13824.

9. Ostrow, B. D., Chen, P., and Chisholm, R. L. (1994) Expression of a myosin regulatory light chain phosphorylation site mutants complements the cytokinesis and developmental defects of *Dictyostelium* RMLC null cells. *J. Cell Biol.* **127,** 1945–1955.
10. Hammer, J. A. III. (1994) Regulation of *Dictyostelium* myosin II by phosphorylation: What is essential and what is important? *J. Cell Biol.* **127,** 1779–1782.
11. Kuczmarski, E. R., Tafuri, S. R., and Parysek, L. M. (1987) Effect of heavy chain phosphorylation on the polymerization and structure of *Dictyostelium* myosin filaments. *J. Cell Biol.* **105,** 2989–2997.
12. Liu, X., Ito, K., Morimoto, S., Hikkoshi-Iwane, A., Yanagida, T., and Uyeda, T. Q. P. (1998) Filament structure as an essential factor for regulation of *Dictyostelium* myosin by regulatory light chain phosphorylation. *Proc. Natl. Acad. Sci. USA* **95,** 14124–14129.
13. Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (Eds.) (1994) Current Protocols in Molecular Biology. Current Protocols, New York.
14. Kubalek, E. W., Uyeda, T. Q. P., and Spudich, J. A. (1992) A *Dictyostelium* myosin II lacking a proximal 58-kD portion of the tail is functional in vivo and in vitro. *Mol. Biol. Cell* **3,** 1455–1462.
15. Shi, S., Lee, R. J., LeBlanc-Straceski, J. M., and Uyeda, T. Q. P. (1999) Role of myosin II tail sequences in its function and localization at the cleavage furrow in *Dictyostelium*. *J. Cell Sci.* **112,** 2195–2201.
16. Egelhoff, T. T., Manstein, D. J., and Spudich, J. A. (1990) Complementation of myosin null mutants in *Dictyostelium discoideum* by direct functional selection. *Dev. Biol.* **137,** 359–367.
17. Ruppel, K. M., Uyeda, T. Q. P., and Spudich, J. A. (1994) Role of highly conserved lysine 130 of myosin motor domain. *J. Biol. Chem.* **269,** 18773–18780.
18. Ruppel, K. M., and Spudich, J. A. (1995) Myosin motor function: Structural and mutagenic approaches. *Curr. Opin. Cell Biol.* **7,** 89–93.
19. Moores, S., Sabry, J. H., and Spudich, J. A. (1996) Myosin dynamics in live *Dictyostelium* cells. *Proc. Natl. Acad. Sci. USA* **93,** 443–446.
20. Yumura, S., and Uyeda, T. Q. P. (1997) Transport of myosin II to the equatorial region without its own motor activity in mitotic *Dictyostelium* cells. *Mol. Biol. Cell* **8,** 2089–2099.
21. Egelhoff, T. T., Titus, M. A., Manstein, D. J., Ruppel, K. M., and Spudich, J. A. (1991) Molecular genetic tools for study of the cytoskeleton in *Dictyostelium*. *Methods Enzymol* **196,** 319–34.
22. Spudich, J. A., and Watt, S. (1971) The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **246,** 4866–4871.
23. Margossian, S. S., and Lowey, S. (1982) Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol.* **85,** 55–71.
24. Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
25. Smith, J. L., Silveira, L. A., and Spudich, J. A. (1996) Activation of *Dictyostelium* myosin light chain kinase A by phosphorylation of Thr166. *EMBO J.* **15,** 6075–6083.
26. Uyeda, T. Q. P., and Spudich, J. A. (1993) A functional recombinant myosin II lacking a regulatory light chain-binding site. *Science* **262,** 1867–1870.
27. Yumura, S., and Kitanishi-Yumura, T. (1992) Release of myosin II from the membrane-cytoskeleton of *Dictyostelium discoideum* mediated by heavy-chain phosphorylation at the foci within the cortical actin network. *J. Cell Biol.* **117,** 1231–1239.
28. Onishi, H., and Wakabayashi, T. (1982) Electron microscopic studies of myosin molecules from chicken gizzard muscle. I. The formation of the intramolecular loop in the myosin tail. *J. Biochem.* **92,** 871–879.
29. Cremo, C. R., Sellers, J. R., and Facemyer, K. C. (1995) Two heads are required for phosphorylation-dependent regulation of smooth muscle myosin. *J. Biol. Chem.* **270,** 2171–2175.
30. Matsu-ura, M., and Ikebe, M. (1995) Requirement of the two-headed structure for the phosphorylation dependent regulation of smooth muscle myosin. *FEBS Lett.* **363,** 246–250.
31. Ito, K., Liu, X., Katayama, E., and Uyeda, T. Q. P. (1999) Cooperativity between two heads of *Dictyostelium* myosin II in in vitro motility and ATP hydrolysis. *Biophys. J.* **76,** 985–992.
32. Flicker, P. F., Peltz, G., Sheetz, M. P., Parham, P., and Spudich, J. A. (1985) Site-specific inhibition of myosin-mediated motility in vitro by monoclonal antibodies. *J. Cell Biol.* **100,** 1024–1030.
33. Brzeska, H., and Korn, E. D. (1996) Regulation of class I and class II myosins by heavy chain phosphorylation. *J. Biol. Chem.* **271,** 16983–16986.
34. Pagh, K., and Gerisch, G. (1986) Monoclonal antibodies binding to the tail of *Dictyostelium discoideum* myosin: Their effects on antiparallel and parallel assembly and actin-activated ATPase activity. *J. Cell Biol.* **103,** 1527–38.
35. Truong, T., Medley, Q. G., and Côte, G. (1992) Actin-activated MgATPase activity of *Dictyostelium* myosin II. Effects of filament formation and heavy chain phosphorylation. *J. Biol. Chem.* **267,** 9767–9772.
36. Warrick, H. M., DeLozanne, A., Leinwand, L. A., and Spudich, J. A. (1986) Conserved protein domains in a myosin heavy chain gene from *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA* **83,** 9433–9437.