The Interaction between the Regulatory Light Chain Domains on Two Heads Is Critical for Regulation of Smooth Muscle Myosin†

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Received October 22, 1999; Revised Manuscript Received December 20, 1999

ABSTRACT: Recent findings have suggested that the interaction between the two heads is critical for phosphorylation-dependent regulation of smooth muscle myosin. We hypothesized that the interaction between the two regulatory light chains on two heads of myosin dictates the regulation of myosin motor function. To evaluate this notion, we engineered and characterized smooth muscle heavy meromyosin (HMM), which is composed of one entire HMM heavy chain and one motor domain truncated heavy chain containing the S2 rod and regulatory light chain (RLC) binding site, as well as the bound RLC (SMDHMM). SMDHMM was inactive for both actin-translocating activity and actin-activated ATPase activity in the dephosphorylated state, demonstrating 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 sufficient for the inhibition of smooth muscle myosin motor activity. When phosphorylated, SMDHMM was activated for both actin-translocating activity and actin-activated ATPase activity; however, these activities were lower than those of double-headed HMM, implying partial release of inhibition by phosphorylation in SMDHMM and/or cooperativity between the two heads of smooth muscle myosin. The present results indicate that the RLC domain is critical for phosphorylation-dependent regulation of smooth muscle myosin motor activity. On the other hand, similar to double-headed HMM, SMDHMM showed both "folded" and "extended" conformations, and the ratio of those conformations is dependent on ionic strength, suggesting that the RLC domain is sufficient to regulate the conformational transition in myosin.

Myosin II is a hexameric molecule composed of two heavy chains, two essential light chains (ELC),[1] and two regulatory light chains (RLC). Each heavy chain consists of a globular catalytic domain, an ELC binding site, an RLC binding site, and a α-helical coiled-coil rod. It is known that the motor activity of smooth muscle and nonmuscle myosin II is activated by phosphorylation of RLC (*1, 2*). The dephosphorylated forms of those myosins have low actin-dependent ATPase activity and are unable to move actin filaments in vitro, whereas the phosphorylated forms are activated in both respects. The location of the RLC on the myosin molecule is at the C-terminal long α-helix portion of the heavy chain in the myosin head region, and the phosphorylation site, Ser-19 on RLC, responsible for activation of myosin motor activity is far from the effector sites, i.e., ATP and actin binding sites in the motor domain.

The requirements for regulation have been elucidated by studies of various proteolytic and expressed subfragments of smooth muscle myosin. Heavy meromyosin (HMM), having two heads but lacking the carboxyl-terminal two-thirds of tail, is regulated by phosphorylation, whereas subfragment 1 (S1), containing a single head and no tail, is constitutively active (*3*). This suggests that the inactivated state of the myosin requires interaction between the two heads or the head and tail. Cremo et al. (*4*) reported that single-headed myosin, a proteolytic fragment of chicken gizzard myosin containing only one head attached to an intact tail, is not regulated by phosphorylation and constitutively active (*4*). The various tail-truncated myosins are studied, and it was found that the truncated myosins forming two-headed structure such as short HMM are regulated by RLC phosphorylation while the myosins failing to form stable two-headed structure become unregulated (*5, 6*). These results indicate that the interaction between the two heads is critical for phosphorylation-dependent regulation.

The myosin head is composed of three domains, i.e., globular motor domain, ELC domain, and RLC domain (*7*). A question is what interactions between those domains in distinct heads are critical for the phosphorylation-dependent regulation. The interaction between two RLC may be the logical consequence of head−head interaction, since the RLC

† This work is supported by NIH Grants AR41653 and HL47530 to M.I. and by an American Heart Association, Massachusetts Affiliate, postdoctoral fellowship to X.L.

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[1] Abbreviations: HMM, smooth muscle heavy meromyosin; RLC, regulatory light chain; ELC, essential light chain; S1, myosin subfragment 1; S2, myosin subfragment 2; SMDHMM, single motor domain HMM; DTE, dithioerythritol; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; his-tag, histidine tag; MLCK, myosin light chain kinase.

10.1021/bi9924617 CCC: $19.00
Published on Web 02/09/2000

domain locates more closely to the joint of the two heads than ELC. Consistent with this notion, a chimeric myosin consisting of the skeletal muscle myosin motor domain and the smooth muscle light chain binding domain plus S2 was found to be regulated by phosphorylation of regulatory light chain. This suggests that the light chain binding region, but not the globular motor domain, is responsible for the regulation (*8*). Disruption of the C-terminal domain (*9*), the central helix (*10*), and the portion of the N-terminus (*11*, *12*) of the RLC hampers the regulation of myosin motor function. Smooth muscle myosin with exchanged skeletal RLC was not regulated by phosphorylation (*13*). Direct chemical cross-linking experiments suggested that the interaction between two RLC is changed by phosphorylation of RLC (*14*). These results support the notion that the interaction between the RLC domain might be important for the regulation of smooth muscle myosin. On the other hand, the ELC appears to be less important for regulation since it can be exchanged with skeletal ELC or removed entirely with little effect on regulation (*15*).

In the present study, we produced and characterized smooth muscle HMM having one entire head and a truncated heavy chain containing the RLC binding domain and S2 in order to evaluate the role of the RLC binding domain on phosphorylation-dependent regulation. The results showed that both enzymatic and motor activities of this construct were well regulated by phosphorylation, indicating that the RLC domain is critical for the head−head interaction of myosin required for the phosphorylation-dependent regulation.

MATERIALS AND METHODS

Materials. Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (*16*). Smooth muscle myosin light chain kinase (MLCK) was prepared from frozen turkey gizzard (*17*). Recombinant calmodulin of *Xenopus* oocyte (*18*) was expressed in *E. coli* as described (*10*).

Generation of the Expression Vectors for Smooth Muscle Myosin Mutants. A baculovirus transfer vector for smooth muscle HMM (6D3) in pBluebac4 was as described previously (*5*). The motor domain deleted construct, S2R, was made as follows. Two *Spe*I sites were created at 5′ side of the initiation codon and at nucleotides 2451−2456 by site-directed mutagenesis. 6D3 having two *Spe*I sites was digested by *Spe*I to remove the sequence corresponding to the motor domain and self-ligated. The Met822 was utilized as a translation initiation site. To facilitate purification, a His-tag sequence was created at the 3′ side of 6D3 by site-directed mutagenesis strategy.

Preparation of Smooth Myosin HMMs. To express double-headed smooth muscle heavy meromyosin (HMM), 200 mL of sf9 insect cells (about 1×10^9), coinfected with three separate viruses expressing HMM heavy chain bearing a C-terminal his-tag and two light chains, was cultured in a large spinner flask containing 600 mL of TMN-FH medium (Sigma) for 3 days. After the cells were harvested by centrifugation, the cell pellets were lysed with 30 mL of Lysis Buffer (0.4 M KCl, 50 mM KP_i, pH 7.5, 5 mM $MgCl_2$, 0.2 mM EGTA, 5 mM β-mercaptoethanol, 2 mM phenylmethanesulfonyl fluoride, 2 mM N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone, 2 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 0.01 mg/mL leupeptin, 1 mg/mL trypsin inhibitor, 0.5% Triton X-100, 1% NP-40, 1 M monosodium glutamate, and 5 mM ATP) with sonication. After centrifugation at 120000*g* for 30 min, the supernatant was incubated with 4 mL of nickel−nitrilotriacetic acid (Ni^2) agarose (Qiagen, Hilden, Germany) in a 50 mL conical tube on a rotating wheel for 3 h at 4 °C. The resin suspension was then loaded on a column (1×10 cm) and was washed with 30 mL of solution A (50 mM NaP_i, pH 7.5, 0.2 M NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.01 mg/mL leupeptin, and 5 mM β-mercaptoethanol). HMM was eluted with 10 mL of solution B (0.2 M imidazole, pH 7.5, 0.2 M NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.01 mg/mL leupeptin, and 5 mM DTE), and 1 mL of each fraction was collected. After SDS−PAGE analysis, the fractions containing HMM were pooled and dialyzed against 50 mM KCl, 30 mM Tris-HCl, pH 7.5, and 1 mM DTT. The purified HMM was stored on ice and used within 2 days. Typically, 0.5 mg of isolated HMM was obtained.

Similar techniques as described above were used for preparing homodimer truncated HMM except that sf9 cells were coinfected with two separate baculoviruses expressing truncated heavy chain having a RLC binding site, S2 region, his-tag, and regulatory light chain, respectively.

To express HMM having the entire S1 on one head and the RLC domain on the other (single motor domain HMM, or SMDHMM), sf9 cells were coinfected with four separate baculoviruses expressing HMM heavy chain without his-tag, the truncated heavy chain (having the RLC binding site, S2 region, and his-tag), and two light chains, respectively, and cultured as described above. Purification of HMM was performed as described above. The eluted mixture containing HMM was pooled and mixed with 0.1 mg/mL actin, 10 units/mL hexokinase, and 5% glucose to eliminate ATP. After incubation for 30 min on ice, the sample was centrifuged at 250000*g* for 15 min to pellet the acto−single-headed HMM complex, while leaving the motor domain truncated myosin in the supernatant. The pellet was washed once with solution C (50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 0.01 mg/mL leupeptin, and 5 mM DTE), and then dissolved in 0.8 mL of 3 mM ATP in solution C. The sample was centrifuged at 250000*g*, for 15 min, and the supernatant was used as SMDHMM. After dialysis against 50 mM KCl, 30 mM Tris-HCl, pH 7.5, and 1 mM DTT, SMDHMM was stored on ice and assayed within 2 days. Normally, about 0.1 mg of SMDHMM could be purified by this method.

Gel Electrophoresis and ATPase Assay. SDS−polyacrylamide gel electrophoresis (PAGE) was carried out on a 7.5−20% polyacrylamide gradient slab gel using the discontinuous buffer system of Laemmli (*19*). Molecular mass markers used were smooth muscle myosin heavy chain (204 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), myosin regulatory light chain (20 kDa), and α-lactalbumin (14.2 kDa). The steady-state ATPase activity of HMM or acto-HMM was determined by measuring the liberated P_i at 25 °C as described previously (*20*).

In Vitro Motility Assay. The in vitro motility assay was performed as described previously (*6*). After thiophospho-

rylation, myosin was attached to a coverslip using a monoclonal antibody, MM9, which recognizes the S2 portion (Ala873−Ser944) of chicken gizzard smooth myosin (*21*). Actin filament velocity was calculated from the movement distance and the elapsed time in successive snapshots. Student's *t* test was used for statistical comparison of mean values. A value of $p < 0.01$ was considered to be significant.

Electron Microscopy. HMM samples in a solution containing 2 mM $MgCl_2$, 0.3 mM DTT, 20 mM Tris-HCl, pH 7.5, 30% glycerol, and 0.4 M KCl or 10 mM KCl were absorbed onto a freshly cleaved mica surface for 30 s. Unbound proteins were rinsed away, and then the specimen was stabilized by brief exposure to uranyl acetate as described (*22*). The specimen was visualized by the rotary shadowing technique according to Mabuchi (*23*) with an electron microscope (Phillips Electronic Instruments, Mahwah, NJ; model EM300) at 60 kV.

RESULTS

Purification of Smooth Myosin SMDHMM. To prepare SMDHMM, sf9 cells were coinfected with four different recombinant baculoviruses encoding smooth muscle HMM heavy chain, a truncated heavy chain containing S2 rod and RLC binding site, essential light chain, and regulatory light chain, respectively. Three different kinds of molecules can be produced, and these are HMM, SMDHMM, and the truncated heavy chain having RLC binding domain + S2. To facilitate the purification of SMDHMM, a His-tag was attached to the C-terminus of the motor domain truncated heavy chain but not HMM heavy chain. It is anticipated that only SMDHMM and the homodimer of the motor domain truncated myosin but not HMM can bind to the Ni^{2+} agarose affinity column. As shown in Figure 1, 128 kDa HMM heavy chain and 35 kDa motor domain deleted heavy chain in addition to the two light chains were detected in the fraction eluted from the affinity column. Significantly more 35 kDa peptide was observed than 128 kDa HMM heavy chain, and this is due to the presence of the homodimer of the motor domain truncated HMM heavy chain (Figure 2). To eliminate the homodimer of motor domain truncated HMM, we utilized the actin binding ability of SMDHMM. The fraction eluted from the affinity column was coprecipitated with actin in the absence of ATP. As expected, the majority of 35 kDa peptide remained in the supernatant, and both 128 kDa HMM heavy chain and 35 kDa peptide were coprecipitated with actin (not shown). The SMDHMM was then dissociated from actin in the presence of ATP and separated from actin by centrifugation (Figure 2). The purified SMDHMM contained 128 kDa HMM heavy chain and 35 kDa motor domain truncated heavy chain with a stoichiometry of 1:(1.04 ± 0.06) (mean ± SD, $n = 4$) determined by densitometric analysis. An electron micrograph of the purified SMDHMM revealed that each molecule has a large globular head and a small RLC binding domain with S2 tail, demonstrating that the purified protein is indeed SMDHMM (Figure 3). The homodimer of the motor domain truncated HMM showed two small globular structures that are observed in one head of SMDHMM, indicating that the small globular structure found in SMDHMM is indeed the RLC binding domain. There was practically no double-headed HMM observed, indicating that the purified sample is free from double-headed HMM.

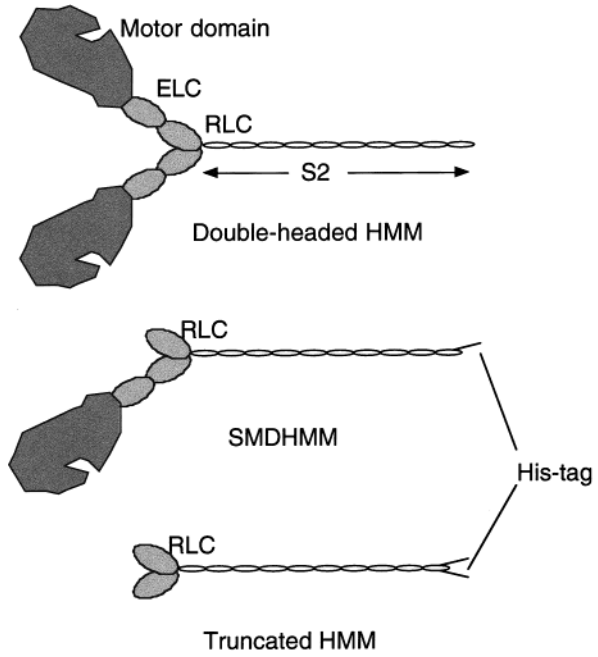

FIGURE 1: Schematic drawing of three species of myosin structure formed in sf9 cells coinfected with four separate recombinant baculoviruses encoding HMM heavy chain, a truncated heavy chain (containing the S2 region and the regulatory light chain binding site), and two light chains, respectively. The double-headed HMM is a hexameric molecule composed of two heavy chains, two ELC, and two RLC. Each heavy chain consists of a motor domain which moves actin filaments, an ELC binding site, an RLC binding site, and an S2 rod. Truncated HMM is missing both pairs of motor domain and ELC domain, whereas SMDHMM is missing one of two pairs of motor domain and ELC domain. A his-tag was attached to the C-terminus of truncated HMM heavy chain to facilitate purification of SMDHMM.

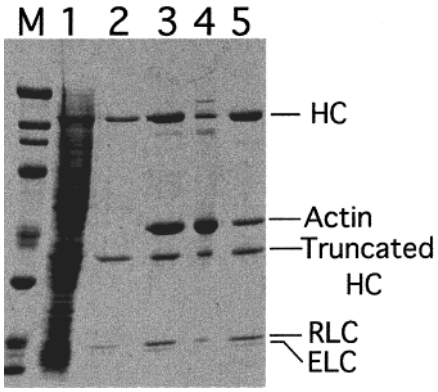

FIGURE 2: Purification of SMDHMM. SMDHMM was purified by Ni^{2+} agarose affinity column chromatography and the actin coprecipitation method as described under Materials and Methods. Lane 1 is the crude extract; lane 2, after Ni^{2+} agarose affinity chromatography; lane 3, coprecipitation with actin. After coprecipitating with actin, SMDHMM was released by addition of 3 mM ATP and separated from actin by ultracentrifugation. Lane 4, pellet; lane 5, supernatant.

When the motor domain truncated heavy chain was solely coexpressed with the light chains in the sf9 cell, no endogenous myosin heavy chain derived from sf9 cells was detected by SDS−PAGE analysis of the sample purified by the Ni^{2+} agarose affinity column (data not shown). This suggests that sf9 myosin heavy chain does not form heterodimer with smooth muscle motor domain truncated heavy chain, and that the endogenous myosin does not interfere with the expression and purification of SMDHMM (Figure 2).

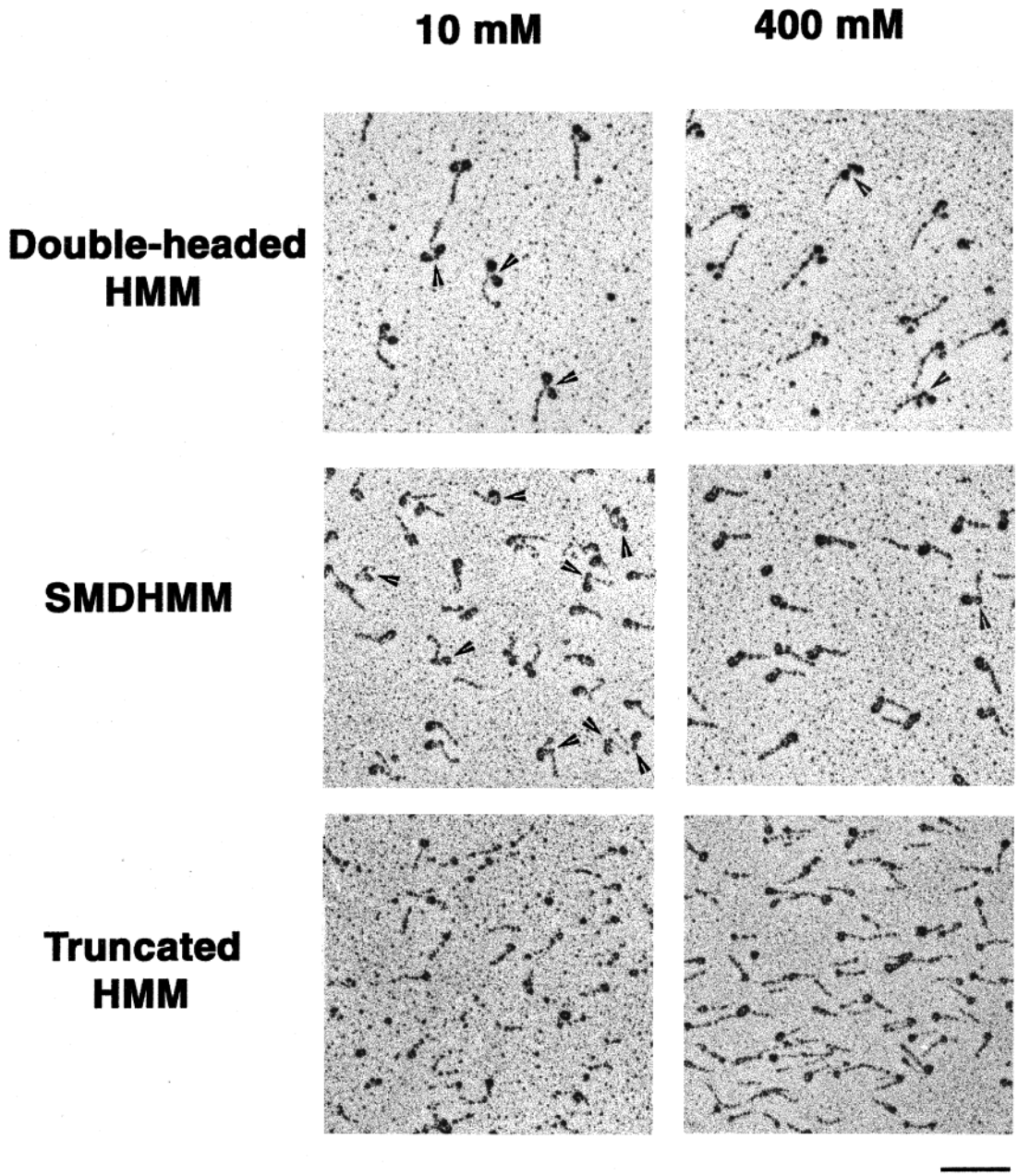

FIGURE 3: Electron micrographs of the purified double-headed HMM, SMDHMM, and the motor domain truncated HMM. HMM samples in a solution containing 2 mM $MgCl_2$, 0.3 mM DTT, 20 mM Tris-HCl, pH 7.5, 30% glycerol, and 0.4 M KCl or 10 mM KCl were absorbed onto a freshly cleaved mica surface for 30 s. Unbound proteins were rinsed away, and then the specimen was stabilized by brief exposure to uranyl acetate as described under Materials and Methods. The rotary-shadowed images of double-headed HMM show two complete heads, whereas the smooth muscle SMDHMM shows a single complete head and a small globular domain comprised of the RLC binding domain. On the other hand, the motor domain truncated HMM lacks a large complete head domain, but contains a small globular domain attached to the tail. The head orientations of double-headed HMM and SMDHMM against the rod were classified into two types: folded and extended forms. The arrowhead indicates the folded form. Note: some double-headed HMM has only 1 folded head, which is counted as 0.5 rather than 1 when calculating the percentage of folded form in Figure 5 ($\times$100000; bars = 100 nm).

ATPase Activities of SMDHMM. Table 1 shows the ATPase activities of SMDHMM and double-headed HMM in various conditions. SMDHMM has normal Ca^{2+}- and K^+-EDTA-ATPase activities, both of which are similar to those of double-headed HMM. In the dephosphorylated state, the basal and actin-activated Mg^{2+}-ATPase activities of SMDHMM are similar to those of double-headed HMM, indicating that SMDHMM is in an inactive state when RLC is dephosphorylated. The Mg^{2+}-ATPase activity of SMDHMM was activated by phosphorylation, although the activation by phosphorylation was less than double-headed HMM. The V_{max} of SMDHMM was about 3 times increased by phosphorylation, while there is little change in K_{actin} by phosphorylation (Table 2). In the phosphorylated state, while the basal Mg^{2+}-ATPase activity of SMDHMM is almost the same as that of double-headed HMM, the actin-activated Mg^{2+}-ATPase activity of SMDHMM is significantly lower than that of double-headed HMM. To examine whether this is due to the difference in K_{actin} or V_{max}, the actin concentration dependence of the ATPase activity was determined. The V_{max} of the phosphorylated SMDHMM was about one-fifth of the double-headed HMM, and the K_{actin} value of SMDHMM is 3.68 times that of the phosphorylated double-headed HMM. Those results suggest that the lower actin-

Table 1: ATPase Activities of SMDHMM and Double-Headed HMM (s^{-1} $Head^{-1}$)

	SMDHMM	double-headed
Ca^{2+}-ATPase[a]	0.68 ± 0.02	0.90 ± 0.03
K^{+}EDTA-ATPase[b]	0.93 ± 0.02	1.5 ± 0.3
Mg^{2+}-ATPase[c]		
Dep., −actin	0.019 ± 0.006	0.014 ± 0.006
Dep., +actin	0.028 ± 0.009	0.025 ± 0.007
P., −actin	0.037 ± 0.003	0.03 ± 0.01
P., +actin	0.060 ± 0.004	0.32 ± 0.05

[a] Ca^{2+}-ATPase activity was measured at 25 °C in 10 mM $CaCl_2$, 0.5 M KCl, 30 mM Tris-HCl, pH 8.5. [b] K^{+}-EDTA ATPase activity was measured at 25 °C in 10 mM EDTA, 0.5 M KCl, 30 mM Tris-HCl, pH 8.5. [c] Assay conditions were 0.01 mg/mL myosin mutant, 0.1 mM ATP, 32 mM KCl, 30 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, with or without 2 mg/mL actin. To measure the activity of the dephosphorylated (Dep.) HMMs, 1 mM EGTA was added, whereas 0.2 mM $CaCl_2$, 15 μg/mL MLCK, and 10 μg/mL calmodulin were added to measure the activity of phosphorylated (P.) HMMs. The actin concentration from the DMSHMM sample in ATPase assay solution was negligibly small (less than 0.005 mg/mL). Results are means ± SD.

Table 2: V_{max} and K_{actin} of MgATPase Activity of SMDHMM and Double-Headed HMM

	dephosphorylated		phosphorylated	
	V_{max} (s^{-1} $head^{-1}$)	K_{actin} (mg/mL)	V_{max} (s^{-1} $head^{-1}$)	K_{actin} (mg/mL)
SMDHMM[a]	0.04 ± 0.01	5.8 ± 1.2	0.12 ± 0.04	6.9 ± 2.0
double-headed[a]	0.04	2.7	0.56	2.1
S1[b]	0.44	5.0	0.44	5.0

[a] Actin-activated ATPase activity was measured at 25 °C in 0.01 mg/mL myosin mutant, 0.1 mM ATP, 30 mM KCl, 2 mM $MgCl_2$, 30 mM Tris-HCl, and various concentrations of F-actin. To measure the activity of phosphorylated myosin, 0.2 mM $CaCl_2$, 15 μg/mL MLCK, and 10 μg/mL calmodulin were added, whereas 1 mM EGTA was added for dephosphorylated myosin. The activity in the absence of actin is subtracted. Results are means ± SD. A computed nonlinear least-squares curve-fitting program was used to estimate the maximum actin-activated ATPase activity (V_{max}) and the apparent dissociation constant for actin (K_{actin}) based on the equation: $V = V_{max}/(1 + K_{actin}/[actin])$. [b] Adapted from ref *3*.

activated ATPase activity of SMDHMM may be due to both lower V_{max} and higher K_{actin}. For both phosphorylated and dephosphorylated forms, the K_{actin} of SMDHMM was significantly higher than that of double-headed HMM, suggesting that the affinity of SMDHMM for actin is lower than that of double-headed HMM. High K_{actin} was also found for S1 (Table 2, and ref *3*).

Actin-Translocating Activity of SMDHMM. To directly evaluate the regulation of SMDHMM motor activity, the actin-translocating activity was measured by an in vitro motility assay. As shown in Figure 4, in the dephosphorylated state, SMDHMM showed no actin-translocating activity, indicating that the motor activity of SMDHMM is completely inhibited. On the other hand, when phosphorylated by MLCK, SMDHMM moved actin filaments at a velocity of 0.08 ± 0.02 μm/s (mean ± SD). More than 90% of the actin filaments were moved. The velocity was about one-third that of double-headed HMM under the same conditions (Figure 4). These results clearly show that the motor activity of SMDHMM is regulated by phosphorylation of RLC.

Conformational Transition of SMDHMM. It is known that the conformation of smooth muscle myosin is sensitive to the change in ionic strength, i.e., a folded conformation

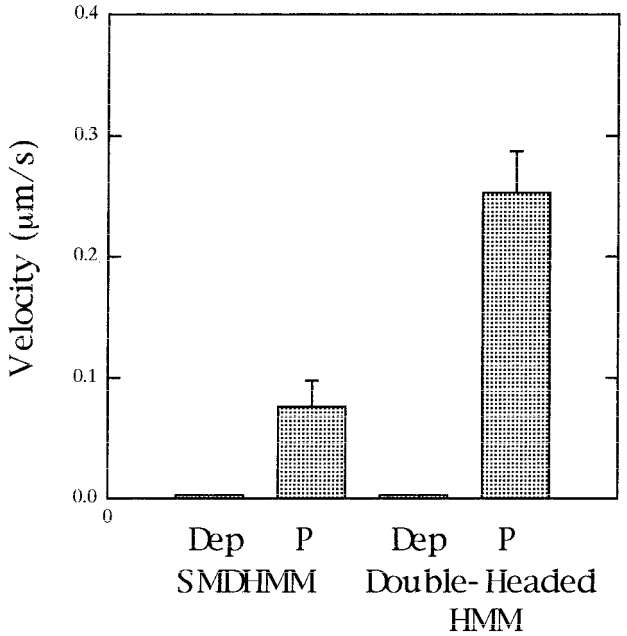

FIGURE 4: Actin sliding velocity of SMDHMM and double-headed HMM. Actin movement was observed in 30 mM KCl, 5 mM $MgCl_2$, 25 mM imidazole, 1 mM EGTA, 1% 2-mercaptoethanol, 0.5% methylcellulose, 4.5 mg/mL glucose, 216 μg/mL glucose oxidase, 36 μg/mL catalase, and 2 mM ATP, pH 7.5 at 30 °C. All values are mean velocities ± SD.

termed 10S at low ionic strength and an extended conformation termed 6S at high ionic strength (*24*−*26*). The conformational transition is regulated by the light chain phosphorylation at physiological ionic strength (*26*−*28*). It has been shown that naturally isolated HMM (*29*, *30*) as well as the recombinant HMM used in this study (*31*) shows the change in conformation that is defined by the head orientation against the S2 rod. Of interest is whether SMDHMM shows the conformational transition. Based upon the head orientation toward the rod, the images were classified into two types: "folded form" with the angle less than 90°, and "extended form" having the angle equal to or more than 90°. The number of molecules was scored in each electronic micrograph, and the percentage of the molecules in "folded form" was calculated. As shown in Figure 5, low ionic strength significantly increased the percentage of "folded form" of SMDHMM, which is very similar to the effects of low ionic strength on double-headed HMM. This result suggests that the RLC domain is sufficient to regulate the conformational transition of myosin, i.e., a folded and an extended form.

DISCUSSION

We have successfully produced SMDHMM having one entire head and one motor domain truncated head. SMDHMM showed clear phosphorylation dependence on its motor activity. The present finding indicates that the interaction between two RLC domains on the distinct heads, and/or between the motor domain on one head and the RLC domain on the other head, but not between two motor domains, is important for the inhibition of smooth muscle myosin motor activity in the dephosphorylated state. The idea that the head−rod junction or S2 portion of the myosin molecule may play a role in the regulation originally came from the finding that the actin-activated ATPase activity of smooth muscle S1 is not regulated by phosphorylation while

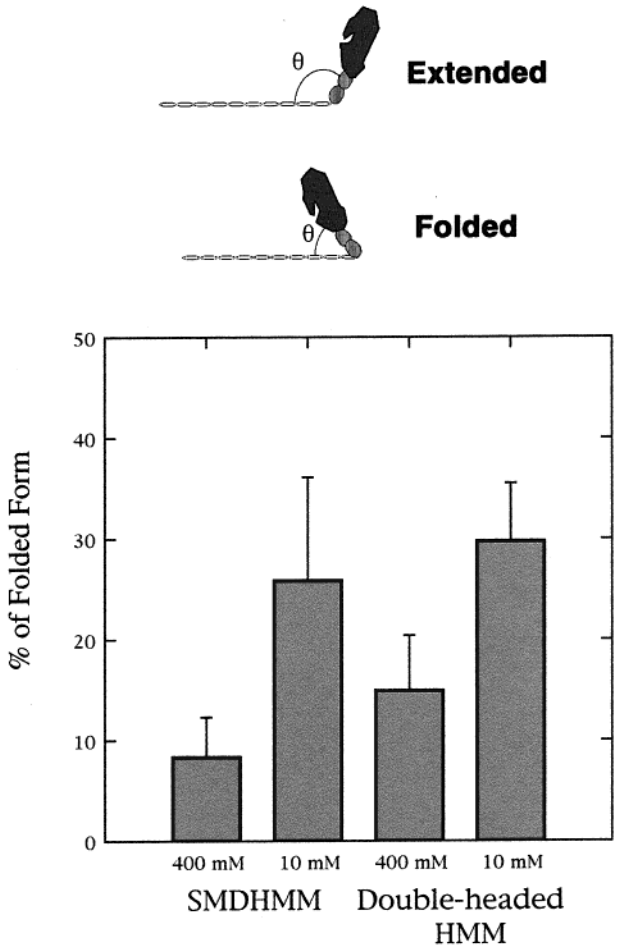

FIGURE 5: Orientation of SMDHMM and double-headed HMM at high and low ionic strengths. Electronic microscopy samples were prepared as described under Materials and Methods. The head orientations against the rod were classified into two types: folded and extended forms. HMM with angle θ between head and rod less than 90° were defined as folded form, whereas those with angle equal to or over 90° were defined as extended form. In each independent electron micrograph, SMDHMM or double-headed HMM molecule was adopted into those two types. The percentage of folded form was calculated. Totally, more than 700 molecules in 20−27 electron micrographs (n) at each condition were scored. All values are mean ± SD. ($P < 0.01$, 400 mM vs 10 mM for both SMDHMM and double-headed HMM.)

that of HMM is (*3*). This notion is further supported by the subsequent findings. The actin-activated ATPase activity of HMM with short tail was regulated to a lesser extent by phosphorylation than that of the HMM with longer tail, suggesting that the tail length is important for the regulation (*15*). By producing the truncated myosins having various lengths of S2, it was found that the truncated myosins forming a single-headed structure are unregulated as in S1 and single-headed myosin (*4*), while those forming double-headed structure are regulated by phosphorylation (*5, 6*). These findings have suggested that two heads are critical for phosphorylation-mediated regulation and imply that head−head interaction is an important feature for inhibition of myosin motor function in the dephosphorylated state. The present results further define the nature of the inter-head interaction.

While the possible interaction between the motor domain and the RLC domain cannot be ruled out, it is more likely that the inter-head RLC−RLC interaction dictates the regulation since: (1) chimeric HMM composed of the skeletal motor domain and smooth light chain binding domain is well regulated by phosphorylation (*8*); (2) according to the 3D structure of the myosin head, the RLC domain is quite distal from the motor domain (*7*); (3) a cryo-atomic force microscopy study showed that the distance between the two heads is increased by phosphorylation of smooth muscle myosin molecules (*32*); and (4) RLC phosphorylation changes chemical cross-linking between two RLC (*14*). The function of S2 is to hold two RLC domains properly and close enough for their interaction, and a specific amino acid sequence in S2 is not required for the regulation (*33*). The role of phosphorylation of Ser-19 would be a change in the inter-head RLC interaction, which releases the inhibition of motor domain function.

While both the actin-activated ATPase activity and the actin-translocating activity of SMDHMM are well regulated by phosphorylation, both the actin-translocating velocity and the ATPase activity of the phosphorylated SMDHMM were significantly lower than those of double-headed HMM. The slower movement is not caused by the introduction of a His-tag at the C-terminal of the heavy chain, since the double-headed HMM with a His-tag at its C-terminal end of the heavy chain has similar motor activity as that of HMM without a His-tag (*34*). The low ATPase and motility activities of SMDHMM are not due to the missing ELC domain in one head. Our preliminary experiments showed that HMM having a truncated neck with two light chains (ELC and RLC) and one intact head produced similar ATPase and motility activities to SMDHMM (Li and Ikebe, unpublished observation), suggesting that ELC is not important in those processes.

The lower motility activity of SMDHMM is at least partly due to the cooperativity of the two entire heads. For smooth myosin, we previously found (*6*) that the actin-translocating velocities of the one-headed truncated myosins are 40−50% of those of two-headed truncated myosins, suggesting that the lower actin-translocating activity of the single-headed myosin construct is due to the cooperativity of the two entire heads. A similar observation was recently reported for *Dictyostelium* myosin II (*35*). The present result of SMDHMM is consistent with the previous finding and further indicates that the cooperativity of motility activity is derived from the motor domain but not RLC domain. Since the actin sliding velocity is determined by d/T_{on} (d, power stroke size; T_{on}, duration of the "on" state) (*36*), it is plausible that the cooperativity between the two heads decreases the T_{on}, and/or increases d, in turn increasing the sliding velocity. Recently, using an optical-trap transducer, Tyska et al. (*37*) measured the unitary displacement and force produced by double- and single-headed smooth muscle myosin. Single-headed myosin produces approximately half the displacement and force of those produced by double-headed myosin during a unitary interaction with actin (*37*), while maintaining T_{on} practically unchanged. This result implies that smooth muscle myosin requires both heads to generate maximal force and motion.

In the phosphorylated state, SMDHMM has significantly lower actin-activated ATPase activity than that of double-headed HMM. The lower actin-activated ATPase activity is not due to the change in the active site since SMDHMM showed normal Mg^{2+}-ATPase, Ca^{2+}-ATPase, and K^{+}EDTA-

ATPase activities. Higher K_{actin} values are observed for SMDHMM, implying the lower affinity of SMDHMM for actin than that of double-headed HMM. High K_{actin} has been reported for smooth muscle myosin S1 (Table 2) (*3*) and *Dictyostelium* myosin II single-headed HMM (*35*), suggesting that the low affinity for actin be due to the single actin binding site in the molecule. On the other hand, the V_{max} of SMDHMM is significantly lower than that of S1 (*6*), and the lower motility activity of SDMHMM would be partly due to the slower ATPase cycle. Taking these findings into account, it is likely that while RLC−RLC interaction is critical for the inhibition of myosin and the phosphorylation abolishes such an interaction, thus activating myosin motor activity, the phosphorylated SMDHMM is still partially inhibited. It was reported recently that when the skeletal light chain binding domain is connected to the smooth muscle myosin motor domain, motor activity is still partially regulated, and it was proposed that the "converter" domain also be involved in regulation (*38*). The "converter" domain may partially play a role in regulation.

It is known that smooth muscle myosin forms two distinct conformations in solution, referred to as 10S (a folded form) and 6S (an extended form) (*24*, *26*), and this is closely correlated to the filament formation of smooth muscle myosin since the 10S conformation cannot form the filaments. The Mg^{2+}-ATPase activity of smooth muscle myosin dramatically decreases upon formation of the 10S form (*39*). Since HMM shows depression at low ionic strength whereas S1 does not (*3*), it was proposed that the head−rod junction is important to the conformational change of myosin. According to an electron microscopic observation of SMDHMM, the present study indicated that the RLC binding domain is important to stabilize a folded conformation at low ionic strength. Therefore, it is plausible that inter-RLC binding domain interaction but not the motor domain is critical for stabilizing a folded conformation. Since the change in the heavy chain sequence at the S1/S2 junction failed to change the conformational transition (*31*), it is less likely that the heavy chain sequence at the head−rod junction itself plays a role in the conformational transition. It was found previously that the basic residues at the N-terminal side of serine 19 (phosphorylation site) of RLC are critical for the formation of a folded conformation (*11*), and it was suggested that an interaction between the heavy chain (presumably acidic residues) and these basic residues of RLC stabilizes a folded conformation. Such acidic residues would be in the RLC binding domain, although it is obscure whether they are in the RLC sequence or the heavy chain sequence.

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BI9924617