Characterization of the Motor and Enzymatic Properties of Smooth Muscle Long S1 and Short HMM: Role of the Two-Headed Structure on the Activity and Regulation of the Myosin Motor†

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ABSTRACT: Truncated mutants of smooth muscle myosin containing various lengths of the S2 portion were expressed in Sf9 cells and purified. Truncated myosin having a heavy chain molecular mass of 128 kDa and larger formed a stable dimer, while 108 kDa myosin remained a monomer. On the other hand, 114 and 110 kDa myosins existed as both monomer and dimer. The enzymatic activity and also the in vitro actin sliding activity of these mutant myosins were measured, and the following findings were obtained. (1) Both the actin sliding activity and the actin-activated ATPase activity showed phosphorylation dependence when myosin forms a dimer while the monomeric form was phosphorylation-independent. This indicates that the interaction between the two heads is operating and critical for the regulation. (2) The actin sliding velocity of the dimer form was twice as large as that of the monomer form, while the actin-activated ATPase activity of the two forms was identical, suggesting that the mechano-chemical efficiency is affected by the interaction between the two heads. (3) The depression of the Mg^{2+}-ATPase activity of myosin at low ionic strength, characteristic of the 6S−10S transition of smooth muscle myosin, is abolished with the monomer form, suggesting that the association of the two heads is critical for the 6S−10S transition.

The activation of smooth muscle and nonmuscle myosin motor requires the phosphorylation of the 20 000 Da light chain subunit of myosin catalyzed by a Ca^{2+}/calmodulin-dependent protein kinase, myosin light chain kinase (MLC kinase)[1] (Hartshorne, 1987; Kamm & Stull, 1989). The phosphorylation sites responsible for the activation are Ser-19 and Thr-18 (Ikebe et al., 1985a, 1986), among which the phosphorylation at Ser-19 is thought to be more physiologically important (Kamm & Stull, 1989). The location of the phosphorylation sites on the light chain is at the C-terminal long α-helix portion of the heavy chain in the myosin head region (Rayment et al., 1993), which is far from the effector sites, i.e., ATP and actin binding sites in the motor domain. A hypothesis was raised (Ikebe et al., 1994) that phosphorylation activates the motor activity of myosin via an intramolecular communication mechanism between the regulatory site and the effector site rather than direct interaction between the two sites.

Although such a mechanism has not been clarified, some progress has been made. Recent structure−function analysis of LC_{20} revealed that the C-terminal portion of LC_{20} is important for regulation by phosphorylation (Trybus & Chatman, 1993; Kamisoyama et al., 1994; Ikebe et al., 1994) and critical for binding to the heavy chain. It is postulated that phosphorylation at the N-terminal region of LC_{20} causes the conformational change of LC_{20} and this change in the conformation is transmitted to the heavy chain via the C-terminal portion of LC_{20} (Ikebe et al., 1994). The idea that the head−rod junction or S2 portion of the myosin molecule may play a role in the regulation is supported by the finding that the actin-activated ATPase activity of smooth muscle S1 is not regulated by phosphorylation while that of HMM is (Ikebe & Hartshorne, 1985b). However, they were unable to identify the portion of S2 crucial for the regulation. Recently, two truncated smooth HMMs with different rod lengths were produced using a baculovirus expression system, and it was suggested that tail length is important for the regulation, based on the observation that the ATPase activity of the short HMM was regulated to a lesser extent by phosphorylation than that of the HMM with a long tail (Trybus, 1994). However, the results could not answer how the S2 portion contributed to the regulation, because the two constructs examined were quite different in length (Met^{1}−Thr^{1027} vs Met^{1}−Glu^{1329}). Recently, we produced truncated MHCs of smooth muscle myosin with various lengths of S2 portions in insect cells (Matsu-ura & Ikebe, 1995). These truncated myosins form dimers dependent on their length of S2 portion, and it is found that the actin-activated ATPase activity is regulated by phosphorylation only when the truncated MHC forms a dimer. However, these studies failed to determine the motor activity (actin sliding activity) of the

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[1] Abbreviations: HMM, heavy meromyosin; S1, myosin subfragment 1; S2, myosin subfragment 2; MLC, myosin light chain; MHC, myosin heavy chain; LC_{20}, 20 000 dalton myosin light chain; LC_{17}, 17 000 dalton myosin light chain; MLCK, myosin light chain kinase; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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truncated myosins. Since it is noted that the actin sliding activity can be decoupled from the ATPase activity (Lowey et al., 1993), it is critical to directly determine the actin sliding activity of myosin to understand the function of the S2 portion on the regulation as well as motor activity.

The purpose of this study is to characterize and determine the motor activity of the truncated smooth muscle myosin mutant molecules which contain various lengths of the S2 portion, thus verifying the function of the S2 portion of smooth muscle myosin molecule for motor activity. Each truncated myosin had a quite unique motor activity according to their length of S2 portion. Results provide further information to understand the mechanism of smooth muscle myosin regulation.

MATERIALS AND METHODS

Protein Preparation. Actin was prepared from rabbit skeletal muscle acetone powder by the method of Spudich and Watt (1971). Myosin light chain kinase was prepared from frozen turkey gizzards (Ikebe et al., 1987). Calmodulin was prepared from bull testes (Walsh et al., 1983). Smooth muscle myosin was prepared from frozen turkey gizzards (Ikebe & Hartshorne, 1985b). HMM and S1 were prepared from myosin by *Staphylococcus aureus* protease digestion (Ikebe & Hartshorne, 1985b).

Coexpression of the Truncated Myosin Heavy Chain with Light Chains in Insect Cells. Five different recombinant baculoviruses (SM944, SM957, SM991, SM1110, and SM1153) were produced to express truncated heavy chains of Met^1-Ser^{944}, Met^1-Gln^{957}, Met^1-Asp^{991}, Met^1-Ser^{1110}, or Met^1-Glu^{1153}, respectively (Matsu-ura & Ikebe, 1995). The cDNA encoding the entire chicken gizzard smooth muscle LC_{17} was obtained from chicken gizzard γZAP cDNA library. After in vitro excision, the insert was subcloned into pT7-7 vector using a unique *Eco*RI site. The clone was then subcloned into the baculovirus transfer vector pBlueBacM (Invitrogen, CA) using a unique *Nhe*I site in the multicloning site which is localized downstream of the polyhedrin promoter. Smooth muscle LC_{20} cDNA (Kamisoyama et al., 1994) was also subcloned into pBlueBacM using an *Nhe*I site. Recombinant baculovirus for each light chain was obtained by the protocols described by O'Reilly et al. (1992). To express truncated smooth muscle myosin, Sf9 cells were coinfected with three separate viruses expressing the heavy chain, LC_{17}, or LC_{20}.

Purification of the Expressed Recombinant Myosins. The cells were harvested 68 h after infection and lysed with 5 volumes of the buffer containing 400 mM KCl, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.2 mM EGTA, 5 mM DTT, 5 mM ATP, 2 mM phenylmethanesulfonyl fluoride, 0.01 mg/mL leupeptin, 0.2 mM N^{α}-tosyl-L-lysyl chloromethyl ketone, 0.2 mM *N*-tosyl-L-phenylalanyl chloromethyl ketone, 0.2 mg/mL ovamucoid trypsin inhibitor, 0.5% Triton X-100, and 1% NP40. After centrifugation at 40000*g* for 1 h, the supernatant was subjected to 30−35% ammonium sulfate fractionation. Actin filament (final concentration 1 mg/mL) was add to this fraction to coprecipitate truncated myosin at 100000*g*. The expressed myosin was eluted from the pellet by adding Mg^{2+}-ATP (1 mM). Approximately 200 μg of the purified myosin was obtained from 1 g of Sf9 cells.

Gel Electrophoresis and ATPase Assay. SDS−polyacrylamide gel electrophoresis was carried out on a 7.5−20% polyacrylamide gradient slab gel using the discontinuous buffer system of Laemmli (1970). Nondenaturing gel electrophoresis was performed according to Persechini et al. (1986). ATPase activity was measured as described previously (Ikebe & Hartshorne, 1985b).

In Vitro Motility Assay. The in vitro motility assay was performed as described previously (Sata et al., 1993) with modification. Myosin sample was thiophosphorylated in 80 mM KCl, 0.2 mM $CaCl_2$, 20 μg/mL MLCK, 10 μg/mL calmodulin, 1 mM adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), and 30 mM Tris-HCl (pH 7.5) at 25 °C for 10 min and was attached to a coverslip by using a monoclonal antibody, MM9, which recognizes the S2 portion ($Ala^{873}-Ser^{944}$) of chicken gizzard smooth muscle myosin (Higashihara et al., 1989). Briefly, 60 μL of MM9 (0.3 mg/mL) in 20 mM Tris-HCl (pH 7.5) was applied to a nitrocellulose-coated coverslip (18 × 24 mm) and then covered by another smaller coverslip (18 × 18 mm), creating a fluid-filled flow cell. After a 5-min incubation on ice, the flow cell was perfused with 120 μL of myosin solution (0.4 mg/mL) followed by BSA solution (0.5 mg/mL BSA, 30 mM KCl, and 20 mM HEPES, pH 7.5) to wash out unbound myosin. Actin filaments labeled with rhodamine−phalloidin (Molecular Probes, Inc., Eugene, OR) were then introduced into the flow cell. The movement of actin filaments was recorded with an inverted fluorescent microscope (Diaphot, Nikon, Japan), an SIT camera (VE 1000 SIT, DAGE MTI), and a videocassette recorder. During a replay of the videotape recording, each video frame was digitized at a rate of 1 frame/s into a 320 × 240 pixel array by a video grabber card (Video Blaster, Creative Labs, CA) installed in a personal computer (Compudyne 614295, COMPUSA, Dallas, TX). Actin filament velocity was calculated from the movement distance and the elapsed time in successive snapshots. The mean velocity for each myosin was calculated from the velocities of 30−40 actin filaments.

Statistical Analysis. All data are presented as a mean value ± SD. The Student's *t*-test was used for statistical comparison of the mean velocities. A value of $p < 0.05$ was considered to be significant.

RESULTS

Coexpression of Truncated Myosin Heavy Chain with Light Chains. Smooth muscle myosin heavy chains with various tail lengths were coexpressed in Sf9 cells with LC_{17} and LC_{20}. As expected by the amino acid residue sequence, SM944, SM957, SM991, SM1110, or SM1153 produced the truncated myosin heavy chain with an apparent molecular mass of 108, 110, 114, 128, or 134 kDa, respectively (Matsu-ura & Ikebe, 1995). The expressed recombinant truncated myosins were almost completely recovered in the extract, suggesting that the expressed myosin fragments are soluble and properly folded. The majority of the extracted recombinant myosin was coprecipitated with F-actin and dissociated from F-actin upon the addition of ATP. Since the ATP-dependent actin binding ability of myosin is a good indication for the functional authenticity of myosin, the result indicates that the expressed myosin retains a native conformation and is functionally active. It should be noted that although the functionally active recombinant myosin fragments can be obtained without coexpression of the light chains, the fractions of the functional molecules were significantly

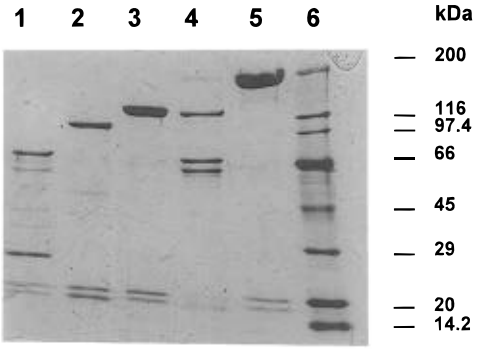

FIGURE 1: SDS−PAGE of the purified truncated smooth muscle myosin. Lane 1, S1; lane 2, 108 kDa myosin; lane 3, 134 kDa myosin; lane 4, HMM; lane 5, myosin; lane 6, molecular weight standards. Molecular weights were estimated using the following standards: smooth muscle myosin heavy chain (200 000), β-galactosidase (116 000), phosphorylase *b* (97 000), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (29 000), trypsin inhibitor (20 100), and lysozyme (14 300).

Table 1: Molar Ratio of the Light Chain to the Heavy Chain

myosin construct	molar ratio (mol/mol)[a]	
	LC_{20}/HC	LC_{17}/HC
myosin	1.04	0.98
134 kDa	1.12	1.03
108 kDa	0.96	1.15

[a] Concentrations of heavy chain and light chains were determined by densitometric scans of SDS−PAGE (Figure 1).

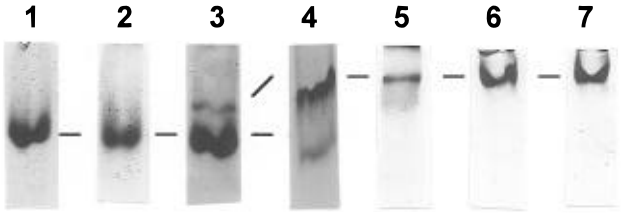

FIGURE 2: Nondenaturing gel electrophoresis of the truncated smooth muscle myosins. Lane 1, S1; lane 2, 108 kDa myosin; lane 3, 110 kDa myosin; lane 4, 114 kDa myosin; lane 5, 128 kDa myosin, lane 6, 134 kDa myosin lane 7, HMM.

increased with coexpression of the light chains (data not shown). The representative preparations of the purified recombinant smooth muscle myosin fragments are shown in Figure 1. The stoichiometry of the heavy chain and light chains of the purified myosin fragments was analyzed by densitometry, and it was found that the myosin fragments were composed of equal moles of the heavy chain and the two light chains (Table 1).

To study whether the expressed truncated myosin forms a double-headed structure or a single-headed structure, the purified myosins were subjected to nondenaturing gel electrophoresis (Figure 2). The 134 or 128 kDa myosin showed a single band whose mobility was similar to that of naturally isolated HMM, indicating that these truncated myosins form a stable double-headed structure. In contrast, the 108 kDa myosin showed a single band with greater mobility which is close to that of naturally isolated S1, showing that these exist as a single-headed form. On the other hand, two bands were observed for both the 110 and 114 kDa myosins. The slower migrating and the faster migrating bands coincided with HMM (Figure 2, lane 7) and S1 bands (Figure 2, lane 1), respectively, thus indicating that these bands represent

Table 2: Relative Amount of the Single-Headed and Double-Headed Forms of Truncated Myosins

truncated myosin	relative amount of myosin (%)[a]	
	double-headed form	single-headed form
HMM	100	0
134 kDa	100	0
128 kDa	100	0
114 kDa	68.2	31.8
110 kDa	11.6	88.4
108 kDa	0	100
S1	0	100

[a] The amounts of double-headed and single-headed forms were determined by densitometric scans of nondenaturing gel electrophoresis (Figure 2).

Table 3: Actin-Activated ATPase Activities of the Truncated Myosins

myosin	actin-activated ATPase activity[a]				
	phosphorylated		dephosphorylated		
	V_{max} (head^{-1} s^{-1})	K_a (μM)	V_{max} (head^{-1} s^{-1})	K_a (μM)	degree of regulation[b]
134 kDa	0.83	90.9	0.05	83.3	16.6
128 kDa	0.77	76.9	0.05	75.8	15.4
114 kDa	0.91	111.1	0.13	100.0	7.0
110 kDa	0.80	105.3	0.77	76.9	1.6
108 kDa	0.77	86.9	0.82	99.0	0.9

[a] Actin-activated Mg^{2+}-ATPase activity was measured at 25 °C in 0.1 mg/mL truncated myosin, 0.3 mM ATP, 30 mM KCl, 30 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, and various concentrations of actin. 0.2 mM $CaCl_2$, 15 μg/mL MLCK, and 10 μg/mL calmodulin were added to measure the activity of phosphorylated myosin, whereas 1 mM EGTA was added for the dephosphorylated one. Mg^{2+}-ATPase activity in the absence of actin is subtracted. [b] Degree of regulation is the ratio of the phosphorylated to dephosphorylated actin-activated ATPase.

the double-headed and the single-headed structure, respectively. For the 110 kDa myosin, the majority of the protein migrated at the single-headed position (Table 2) while the double-headed form was dominant for the 114 kDa myosin (Table 2). These data are consistent with gel filtration results in our previous study, demonstrating that the residues Gln^{945}−Ser^{1110} are critical for the formation of the double-headed structure of myosin (Matsu-ura & Ikebe, 1995). To study the effect of phosphorylation of LC_{20} on the dimer/monomer ratio, the 114 kDa myosin was thiophosphorylated under the same condition as that for the in vitro motility assay and subjected to nondenaturing gel electrophoresis. The relative amount of the double-headed form did not significantly change by phosphorylation (data not shown).

Actin-Activated ATPase Activity. Table 3 summarizes the actin-activated Mg^{2+}-ATPase activities of the truncated myosins. The ATPase activities of the 134 and 128 kDa myosins were markedly activated by phosphorylation as was naturally isolated HMM (Ikebe & Hartshorne, 1985b). In contrast, the ATPase activity of the 108 kDa fragment showed no dependence of phosphorylation, and the activity was virtually the same as those of phosphorylated 134 and 128 kDa myosins. On the other hand, the ATPase activities of the 110 and 114 kDa myosins were dependent on phosphorylation, but the phosphorylation dependence was not as extreme as was found for the 134 and 128 kDa myosins. The phosphorylation dependence was more pronounced for the 114 kDa myosin than for the 110 kDa myosin. Apparently, the change in the phosphorylation

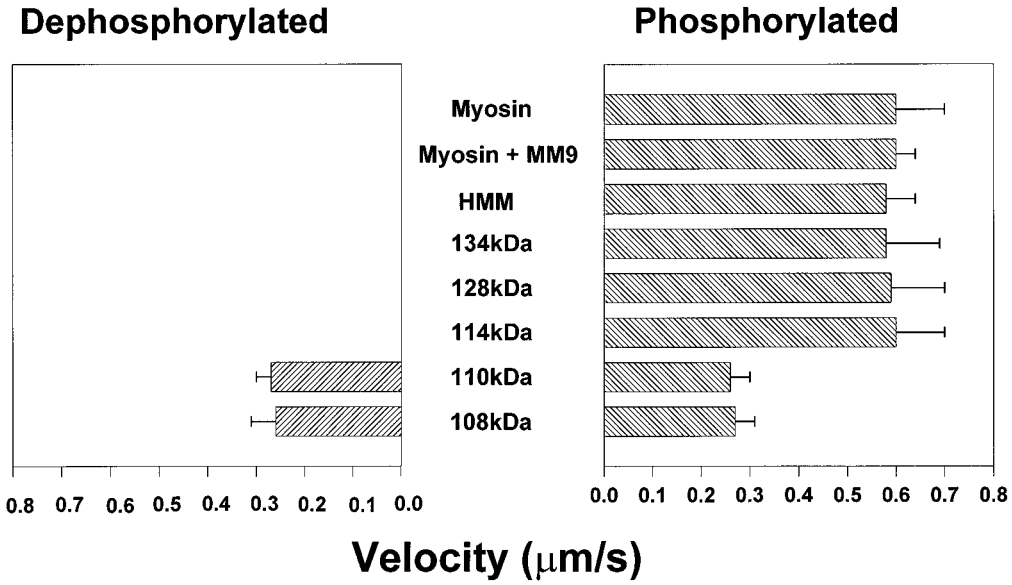

FIGURE 3: Sliding velocity of actin filaments on the truncated smooth muscle myosins. Velocity was measured in the assay buffer (30 mM KCl, 5 mM $MgCl_2$, 25 mM imidazole, 1 mM EGTA, 1% 2-mercaptoethanol, 0.5% methylcellulose, 4.5 mg/mL glucose, 216 μg/mL glucose oxidase, 36 μg/mL catalase, and 2 mM ATP, pH 7.5) at 25 °C as described under Materials and Methods.

dependence is correlated with the fraction of the double-headed form (Table 2), indicating that the phosphorylation dependence of the actin-activated ATPase activity requires the double-headed structure of myosin. On the other hand, the dissociation constant for actin (K_a) did not show any dependence on the length of the S2 portion, suggesting that actin binding to the individual head is not affected by dimer formation.

Actin Sliding Velocity. To evaluate more directly the motor activity of the various truncated mutants of smooth muscle myosin, the actin filament sliding velocity induced by myosin molecules was measured. When the truncated myosin was fixed directly to the nitrocellulose membrane, only sporadic movements were observed for these truncated myosins but this was overcome by fixing the myosins using a monoclonal antibody, MM9, which recognizes the S2 portion of the myosin molecule (Higashihara et al., 1989). The results of the in vitro motility assay are summarized in Figure 3. More than 90% of the actin filaments in a visual field continued to move smoothly and unidirectionally at a constant velocity when MM9 antibody was used. Because there was no significant difference between the actin velocities on native myosin fixed with and without MM9 (Figure 3), the use of this antibody did not affect the actin sliding velocity of the system.

There are two important findings in Figure 3. First, the truncated myosins having 114 kDa and larger molecular mass exhibited complete phosphorylation-dependent motility activity. In contrast, the motility activity of the truncated myosin below 110 kDa was independent of phosphorylation. This is consistent with the actin-activated ATPase activity (Table 3), and indicates that the double-headed structure is critical for the phosphorylation-dependent motor activity. Second, the motility activities of 110 and 108 kDa myosins were significantly lower than those of the phosphorylated myosin fragments having a double-headed form; i.e., the actin sliding velocity of the single-headed form was 40−50% of that of the double-headed form. The results suggest that the actin sliding activity of the double-headed form is twice as large as that of the single-headed form.

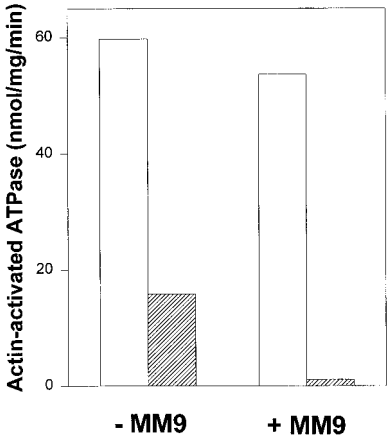

FIGURE 4: Actin-activated ATPase activity of 114 kDa myosin in the absence and in the presence (0.02 mg/mL) of MM9. Actin-activated Mg^{2+}-ATPase activity was measured at 25 °C in 0.1 mg/mL myosin, 0.3 mM ATP, 30 mM KCl, 30 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, and 1.2 mg/mL actin. 0.2 mM $CaCl_2$, 15 μg/mL MLCK, and 10 μg/mL calmodulin were added to measure the activity of phosphorylated myosin (open columns), whereas 1 mM EGTA was added for the dephosphorylated myosin (hatched columns).

To study the effect of anti-S2 antibody, MM9, on dimer formation, the actin-activated ATPase activity of 114 kDa myosin was measured in the absence and the presence of MM9. In the presence of this antibody, the activity of the dephosphorylated but not phosphorylated 114 kDa myosin was markedly decreased (Figure 4), suggesting that the antibody favored dimer formation by binding to the coiled-coil helix of S2 portion, thus eliminating the phosphorylation-independent activity due to monomer myosin.

KCl Dependence of Mg^{2+}-ATPase Activity for the Truncated Myosins. It has been shown that the ATPase activities of smooth muscle myosin are sensitive to changes in ionic strength; i.e., the activity markedly decreases in low ionic strength, corresponding to a shift in myosin conformation from 6 S to 10 S (Ikebe et al., 1983). HMM has been shown to retain this property whereas S1 does not; hence, it has been hypothesized that the head−rod hinge structure is responsible for the decrease in the ATPase activity in low

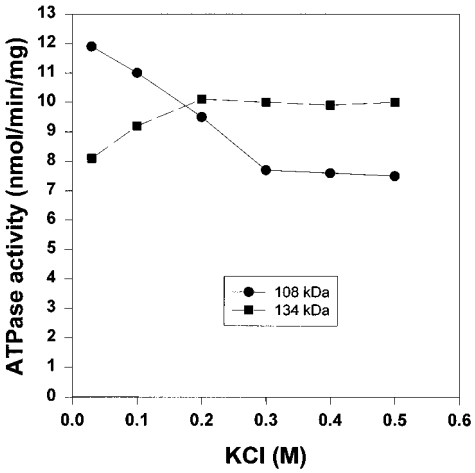

FIGURE 5: KCl dependence of the Mg^{2+}-ATP activity of the single-headed myosin fragment and the double-headed fragment. Conditions: 0.1 mg/mL truncated myosin, 0.1 mM ATP, 30 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, and various concentrations of KCl at 25 °C. (■) 134 kDa myosin; (●) 108 kDa myosin.

ionic strength. To identify the effect of the S2 portion of myosin on this conformational change, the KCl dependence of the MgATPase activities for 108 and 134 kDa myosins was studied (Figure 5). The activity of 134 kDa myosin was decreased with the reduction in ionic strength, while that of 108 kDa myosin was increased as the ionic strength was reduced as was found for the naturally isolated S1 (Ikebe & Hartshorne, 1985b).

DISCUSSION

The smooth muscle myosin heavy chains and two light chains were coexpressed. Almost all expressed myosin heavy chain was stoichiometrically associated with the two light chains and functionally active. On the other hand, the large fraction of myosin heavy chain was insoluble when the heavy chain was not coexpressed with the light chains. The results suggest that the binding of the light chains to the heavy chain stabilizes the conformation of the myosin molecule. Previously, it was reported that the lack of coexpression of LC_{17} does not markedly change the fraction of functional molecules (Trybus, 1994); therefore, the coexpression of LC_{20} may be more essential for stabilization of the native conformation of myosin. It was shown that removal of LC_{20} from naturally isolated myosin diminished the motility activity but the activity was restored by addition of the exogenous LC_{20} (Trybus et al., 1994), suggesting that the myosin conformation is rather stable in the postfolded protein even in the absence of LC_{20}. Therefore, it is plausible that LC_{20} stabilizes the functional myosin conformation during protein synthesis, i.e., the protein folding process.

The objective of this study is to identify the function of the S2 portion of the myosin molecule on smooth muscle myosin. Three important findings were made from this study. First, the obtained results clearly showed that the two-headed structure is necessary for the phosphorylation-dependent myosin motor activity. Cremo et al. (1995) recently showed that single-headed myosin produced by proteolytic digestion showed phosphorylation-independent motor activity, suggesting that the double-headed structure is critical for the regulation although the possible effect of the remaining rod on the regulation may not be excluded. The results obtained here are consistent with this observation and, furthermore, indicate that even though the myosin heavy chain length and the polypeptide composition of myosin are exactly the same, the phosphorylation-dependent regulation operates only when smooth muscle myosin forms a dimer structure (Tables 2 and 3).

Determination of the motility activity of smooth muscle myosin with a short tail has not been successful (Trybus, 1994), presumably due to the steric hindrance problem. We also found that the direct attachment of the truncated myosin to the nitrocellulose membrane does not produce continuous stable actin movement. This problem was overcome by using a specific monoclonal antibody recognizing the S2 portion of the molecule. The use of antibodies for in vitro motility assay has been used by other investigators (Cuda et al., 1993; Trybus, 1994; Waller et al., 1995), and it has been shown that antibodies do not alter the actin sliding velocity. This is also the case in this study and thus a useful method to analyze the activity of myosin motors with short tails.

Second, the results indicate that the actin sliding velocity of the single-headed molecule, i.e., long S1, is approximately half of that of the double-headed molecule, i.e., HMM (Figure 3). The tail length of the myosin molecule itself is not critical for the sliding velocity since myosin molecules having 114 kDa and larger heavy chain mass show virtually identical actin sliding velocity, and the velocities of the 110 and the 108 kDa myosins are also the same. Of interest is that the actin-activated ATPase activities of the single-headed myosins are virtually the same as those of the double-headed myosins. It is generally accepted that the actomyosin ATPase cycle is divided into two states, i.e., the weak binding state and the strong binding state (Eisenberg & Hill, 1985; Siemankowski et al., 1985). While the rate-limiting step of the ATPase reaction is thought to be in the weak binding state (Rosenfeld & Taylor, 1984), the sliding velocity is related to the force-generating strong bound state. Therefore, the present results suggest that the kinetic process of the power-stroke state is facilitated due to the formation of the double-headed structure, while the weak binding state is unchanged. This needs to be proven by further detailed kinetical study. Since the ATP consumption of the single-headed and the double-headed myosin fragments is the same, the difference in the motility activity would imply the difference in mechano-chemical efficiency between the two forms.

It should be noted that while significant actin-activated ATPase was observed for the dephosphorylated 114 kDa myosin, no motility was observed for the dephosphorylated 114 kDa myosin (Table 3, Figure 3). It is plausible since MM9 binds to the S2 portion of myosin molecule, it would stabilize the dimer structure. In addition to this, the motility activity of phosphorylated 114 kDa myosin was exactly the same as the dimer forms of myosins but larger than the monomer form. These results suggest that the 114 kDa myosin in motility assay conditions is predominantly a dimer, in contrast to the results in Table 2 where 30% of the 114 kDa myosin exists as a monomer. This puzzle was clarified by the finding that an anti-S2 antibody, MM9, favored dimer formation of 114 kDa (Figure 4). Presumably, most of the 114 kDa molecules existed as dimers in the motility assay after being attached to a coverslip via the antibody, and the concentration of the single-headed form was not be enough to support actin sliding as was previously discussed for

skeletal myosin (Uyeda et al., 1990).

Third, the present results suggest that the double-headed structure is also critical for the conformational transition of smooth muscle myosin known as the 6S−10S transition (Suzuki et al., 1978). It is known that smooth muscle myosin can form two distinct conformations in solution, referred to 10 S and 6 S (Suzuki et al., 1978; Trybus et al., 1982; Onishi & Wakabayashi, 1982; Craig et al., 1983). The Mg^{2+}-ATPase activity of myosin alone dramatically decreases upon formation of the 10S form (Ikebe et al., 1983). Subsequently, it was proposed that the head−rod junction is important to express the conformational change of myosin since the Mg^{2+}-ATPase activity of S1 does not show the depression at low ionic strength, in contrast to HMM (Ikebe & Hartshone, 1985b), and the digestibility at the head−rod junction is markedly changed upon formation of the 10S conformation (Ikebe & Hartshorne, 1984; Onishi & Watanabe, 1984). Our results clearly show that the ATPase activity of 108 kDa myosin (long S1) is not depressed at low ionic strength, while that of 134 kDa myosin (double-headed form) is. The tail length of the 108 kDa myosin is much longer than that of the naturally isolated S1, whose apparent molecular mass of the heavy chain is 94 kDa, and a significant portion of the S2 region is included in the 108 kDa myosin heavy chain, thus containing the entire head−rod hinge region. Therefore, it is plausible that the critical factor determining the conformational change responsible for the depression of Ma^{2+}-ATPase activity at low ionic strength is the association of the two heads.

The information obtained in this study strongly suggests that there is the interaction between the two heads of smooth muscle myosin and this plays a critical role in the characteristic nature of the regulation and function of smooth muscle myosin.

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