A Specific Amino Acid Sequence at the Head−Rod Junction Is Not Critical for the Phosphorylation-Dependent Regulation of Smooth Muscle Myosin†

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ABSTRACT: It has been suggested that the structure at the head−rod junction of smooth muscle myosin is important for the phosphorylation-mediated regulation of myosin motor activity. To investigate whether a specific amino acid sequence at the head−rod junction is critical for the regulation, three smooth muscle myosin mutants in which the sequence at the N-terminal end of S2 is deleted to various extents were expressed in Sf9 cells; 28, 56, and 84 amino acid residues, respectively, at the position immediately C-terminal to the invariant proline (Pro849) were deleted, and the S1 domain was directly linked to the downstream sequence of the rod. The mutant myosins were expressed, purified, and biochemically characterized. All three myosin mutants showed a stable double-headed structure based upon electron microscopic observation. Both the actin-activated ATPase activity and the actin translocating activity of the mutants were completely regulated by the phosphorylation of the regulatory light chain. The actin sliding velocity of the three mutant myosins was the same as the wild-type recombinant myosin. These results indicate that a specific amino acid sequence at the head−rod junction is not required for the regulation of smooth muscle myosin. The results also suggest that there is no functionally important interaction between the regulatory light chain and the heavy chain at the head−rod junction.

Smooth muscle and nonmuscle myosin motor activities depend on the phosphorylation of the regulatory light chain at Ser19, catalyzed by Ca^{2+}-calmodulin-dependent myosin light chain kinase (*1*−*5*). The regulatory light chain is associated at the C-terminal end of S1[1] which localizes distal from both the ATP binding site and the actin binding site (*6*). Of interest is how the phosphorylation at Ser19 can activate the myosin motor activity. Based upon the recent 3D structure of the myosin head (*6*), it is unlikely that the phosphate moiety of the side chain of Ser19 directly interacts with the catalytic site and/or the actin binding site which are localized toward the top of the myosin head. This leads to the hypothesis that the change in the conformation of LC20, induced by phosphorylation, is transmitted to the motor effector sites via intersubunit communication, which would be critical for the regulation of myosin motor activity (*7*). Several reports using various probes such as limited proteolysis (*8*−*10*), fluorescence anisotropy (*11*), and electron microscopy (*12*−*14*), have suggested that regulatory light chain phosphorylation induces a change in conformation at the head−rod junction of myosin. While the mechanism by which phosphorylation regulates the motor activity of myosin is still obscure, it has been postulated that the interaction between the two heads of myosin may be involved in the phosphorylation-mediated activation mechanism. This was originally suggested by the fact that the actomyosin-activated ATPase activity of HMM- but not S1-containing intact regulatory light chain is regulated by phosphorylation (*9*). This view was further supported by a recent finding that the motor activities of the single-headed myosin (*15*) and single-headed long S1 (*16*, *17*) are not regulated by phosphorylation but double-headed short HMM are (*16*, *17*). Quite recently, it was also found that the motor activity of the chimeric myosin, which consists of the skeletal globular motor domain and smooth muscle light chain associated domain plus S2, is regulated by the phosphorylation of the regulatory light chain (*18*). This suggests that the light chain binding domain confers the phosphorylation-dependent regulatory activity of smooth muscle myosin. However, Trybus et al. (*19*) recently showed that the S2 portion of the myosin molecule plays a important role in the phosphorylation-dependent regulation of smooth muscle myosin. They introduced a leucine-zipper motif into the S2 portion of the myosin molecule and found that the produced mutant myosin became unregulated by phosphorylation, suggesting that the S2 portion may play an important role in the regulation of smooth muscle myosin motor function. Since the bend at the head−rod junction of smooth muscle, but not striated muscle, myosin seems to be changed by phosphorylation (*14*, *20*−*22*), it is plausible that the specific amino acid sequence at the head−rod junction, or S2, is critical for the regulation,

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′, *N*′-tetraacetic acid; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; S2, myosin subfragment 2; S1, myosin subfragment 1; HMM, heavy meromyosin.

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either by changing the flexibility upon phosphorylation or by interacting with the head portion of the myosin molecule. A question is whether the unique amino acid sequence at the head−rod junction in smooth muscle myosin is critical for the phosphorylation-dependent regulation of its motor activity.

In the present study, we produced smooth muscle myosins in which the amino acid residues at the head−rod junction in S2 were deleted to various extents, and the S1 portion of the molecule was linked directly to the downstream sequence of the myosin rod. These mutant myosins were expressed in Sf9 cells, isolated, and characterized in order to investigate whether this region of the molecule plays a critical role in the regulation of myosin motor function.

MATERIALS AND METHODS

Materials. Restriction enzymes and modifying enzymes were purchased from New England Biolab (Beverly, MA). Smooth muscle myosin was prepared from frozen turkey gizzards as described (*23*). Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (*24*). Smooth muscle myosin light chain kinase was prepared from frozen turkey gizzards (*25*). Recombinant calmodulin of *Xenopus* oocyte (*26*) was expressed in *E. coli* as described (*27*).

Expression of Recombinant Smooth Muscle Myosin Mutants. Smooth muscle myosin heavy chain cDNA was obtained from chicken gizzard as described previously (*16*). The cDNA encoding 1298 amino acid residues of myosin heavy chain was subcloned into pBluscript SKII(+) vector, and *Spe*I sites were introduced before the initiation codon and after the stop codon, respectively. Site-directed mutagenesis was performed using this construct as described previously (*7*, *28*). *Nde*I sites were introduced at the 851th and 879th codons, 851th and 907th codons, or 851th and 935th codons, respectively. Each cDNA containing two *Nde*I sites was subjected to restriction digestion with *Nde*I to remove the sequence corresponding to a part of myosin S2. The produced myosin mutants lack amino acid residues Gln852−Ala879 (ΔA), Gln852−Glu907 (ΔAB), and Gln852−Ala935 (ΔABC), respectively. The truncated cDNA was purified by agarose gel electrophoresis and then self-ligated. After confirming the mutation by direct sequencing analysis (*29*), the mutant myosin heavy chain cDNA was excised from the vector by *Spe*I digestion and subcloned into a pBlueBac4 baculovirus transfer vector (Invitrogen) using the unique Nhe1 site in the polylinker region of the vector. The orientation of the myosin heavy chain cDNA in the vector was confirmed by restriction mapping as well as by direct sequencing analysis. The baculovirus transfer vectors of smooth muscle light chains were produced as described (*17*). Recombinant baculoviruses for the heavy chain and the light chains were produced according to the protocols described by O'Rielly et al. (*30*). The recombinant virus expressing myosin heavy chain (Met1−Ser1110) (*17*) was used to prepare the wild-type truncated myosin. To express smooth muscle myosin mutants, Sf9 insect cells were coinfected with three separate viruses expressing the heavy chain and two light chains. The recombinant smooth muscle myosin was purified as described previously (*17*) with slight modification. In brief, the cells were harvested 72 h after infection. The cells were lysed, and the cell homogenate was centrifuged as described (*17*); 1 M glucose, 20 units/mL hexokinase, and 0.3 mg/mL F-actin were added to the supernatant, and the expressed myosin was coprecipitated with actin by centrifugation (100000*g* for 1 h). The pellets were washed with 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, 10 mM $MgCl_2$, 0.5 mM EGTA, and 5 mM DTT and then centrifuged (100000*g* for 15 min) in the presence of 5 mM ATP to dissociate myosin from actin. The supernatant was dialyzed against 20 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, and 1 mM DTT to remove ATP. The sample was centrifuged (10000*g* for 10 min) to remove endogenous Sf9 myosin.

Determination of Myosin Motor Function. Actin-activated ATPase activity was measured at 25 °C in assay mixture containing 0.1 mg/mL myosin, 10 mM $MgCl_2$, 30 mM KCl, 0.2 mM ATP, and 30 mM Tris-HCl, pH 7.5, with various concentrations of F-actin. The ATPase activity of myosin or actomyosin was determined by measuring the liberated ^{32}P as described previously (*23*). SDS−PAGE was carried out on 7.5−20% polyacrylamide gradient slab gels by using the discontinuous buffer system of Laemmli (*31*). 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).

Actin translocating velocity was measured by in vitro motility assay as described previously (*17*). The mean velocities for each myosin mutant were calculated from the velocities of 30−40 actin filaments. 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. Myosin 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 were absorbed onto a freshly cleaved mica surface for 30 s. Unbound protein was rinsed away, and then the specimen was stabilized by brief exposure to uranyl acetate as described (*32*). The specimen was visualized by the rotary shadowing technique according to Mabuchi (*33*) with an electron microscope (Phillips Electronic Instruments, Mahwah, NJ; Model EM300) at 60 kV.

RESULTS

Expression and Purification of Smooth Muscle Myosin S2 Deletion Mutants. It is known that myosin rod forms an α-helical coiled-coil structure and shows a characteristic seven residue repeat pattern with hydrophobic residues in the first and fifth positions which forms the hydrophobic core of the coiled-coil (*34*). Four heptapeptide repeats in the myosin rod show a 28 residue periodicity with alternating bands of positive and negatively charged residues (*35*−*37*). The smooth muscle myosin rod retains a 28 residue repeat starting with Pro 849 at position 15 of the 28 residue repeat (*38*). To investigate whether a specific amino acid sequence at the head−rod junction is critical for the phosphorylation-mediated regulation of smooth muscle myosin motor function, we deleted 1, 2 or 3, 28 amino acid repeats at the head−rod junction of S2, termed ΔA, ΔAB, and ΔABC myosin, respectively (Figure 1). Previously, it was shown that the

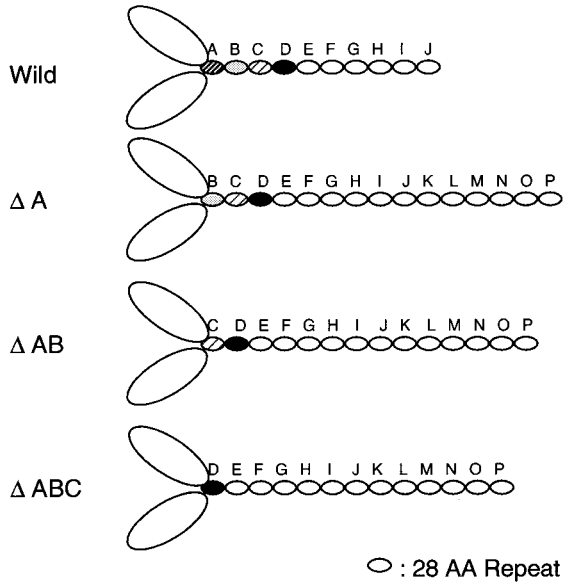

FIGURE 1: Schematic drawing of the smooth muscle myosin mutants in which the amino acid residues at the head−rod junction are deleted.

two-headed myosin structure is critical for the regulation of smooth muscle myosin motor activity (*16*, *17*). To produce a myosin fragment forming a stable two-headed structure for the S2 deletion mutants, the smooth muscle myosin heavy chain cDNA encoding Met1−Val1298 was made by introducing a stop at codon 1299 by site-directed mutagenesis. The expressed smooth muscle myosin fragment contains 16, 28 residue repeats with a calculated molecular mass of 150 kDa. This cDNA construct was used as a template for site-directed mutagenesis to produce the S2 deletion mutants. The deleted amino acid residues adjacent to the invariant proline are shown in Figure 2.

Smooth muscle myosin heavy chain mutants were coexpressed in Sf9 cells with two light chains. Most of the expressed truncated myosin mutants were solubilized in the extract, but a portion of the expressed heavy chain was insoluble. Repeated extraction did not increase the soluble myosin fraction, suggesting that the insoluble myosin was improperly folded. Functionally active mutant HMM was coprecipitated with F-actin in the absence of ATP and dissociated from actin upon addition of ATP (Figure 3A). Approximately 1 mg of purified myosin was obtained from 1 L of culture. Sf9 cells also expressed endogenous myosin, with 200 kDa heavy chain, and this was completely removed by centrifugation in the presence of 10 mM $MgCl_2$ and 50 mM KCl. Figure 3B shows SDS−PAGE of the expressed and purified myosin mutants in which the S2 portion at the head−rod junction was deleted to various extents. The apparent molecular masses of ΔA, ΔAB, and ΔABC myosins estimated from the relative mobility to molecular mass standards were 135, 132, and 129 kDa, respectively. This is consistent with the expected molecular mass of the three deletion mutants, indicating that the examined myosins were indeed the designed mutant forms. This was further supported by observation using electron microscopy. As shown in Figure 4, ΔA, ΔAB, and ΔABC myosins all showed a double-headed structure. Virtually no full-length myosin molecules were observed as judged from the tail length of myosin, suggesting that there was practically no contamination of endogenous myosin. Consistent with this observation, there was no detectable 200 kDa band corresponding to endogenous Sf9 myosin in the purified sample analyzed by SDS−PAGE (Figure 3A).

V_{max} of the Actin-Activated ATPase Activity of Smooth Muscle Myosin Mutants. The actin-activated ATPase activity of the smooth muscle myosin S2 deletion mutants was measured as a function of F-actin concentration. The ATPase activity in the absence of F-actin was subtracted from the value in the presence of F-actin, and the V_{max} was estimated from the double-reciprocal plots of the F-actin dependence of ATPase activity. For all of the myosin mutants tested (ΔA, ΔAB, and ΔABC), the V_{max} of the actin-activated ATPase activity was significantly enhanced by phosphorylation of the regulatory light chain (Table 1). The apparent dissociation constant for actin, K_a, of the mutant myosins was similar to the naturally isolated smooth muscle HMM and the recombinant wild-type truncated myosin fragment which contained intact S2 amino acid sequence (data not shown). The result clearly indicates that the specific amino acid sequence at the head−rod junction or S2 is not critical for the light chain phosphorylation-dependent regulation of the actin-activated ATPase activity of smooth muscle myosin.

Actin Translocating Activity of Smooth Muscle Myosin Mutants. To evaluate more directly the motor activity of the various myosin mutants, the actin translocating velocity of the deletion mutants as well as the wild-type recombinant myosin was measured by an in vitro motility assay. Figure 5 shows the actin translocating velocity of the recombinant smooth muscle myosin fragments. The in vitro motility activity of the smooth muscle myosin S2 deletion mutants was completely regulated by the light chain phosphorylation, and no motility activity was observed for the dephosphorylated forms. The actin translocating velocity of all three phosphorylated myosin mutants in which S2 was deleted to various extents was indistinguishable from that of the wild-type recombinant myosin fragment. These results were consistent with the actin-activated ATPase activity and indicated that the specific amino acid sequence at the head−rod junction is not required for the regulation of smooth muscle myosin motor activity. The result also shows that (1) the length of the S2 portion and (2) the primary structure of the S2 portion are not critical for the actin-translocating velocity of the myosin molecule.

DISCUSSION

Smooth muscle myosin mutants in which the N-terminal end of the S2 sequence was deleted to various extents were expressed and isolated. The isolated mutant myosins showed complete phosphorylation dependence for their actin-activated ATPase and actin translocating activity. It was shown previously that single-headed myosin (*15*) and long S1 (Met1−Gln957) (*16*, *17*) show phosphorylation-independent constitutively active motor activity, while double-headed short HMM (Met1−Asp991) is completely phosphorylation-dependent and light chain phosphorylation is required for the actin-activated ATPase activity (*16*), as well as in vitro motility activity (*17*), indicating that the interaction between the two heads is critical for the inhibition of smooth muscle motor activity which is released by regulatory light chain

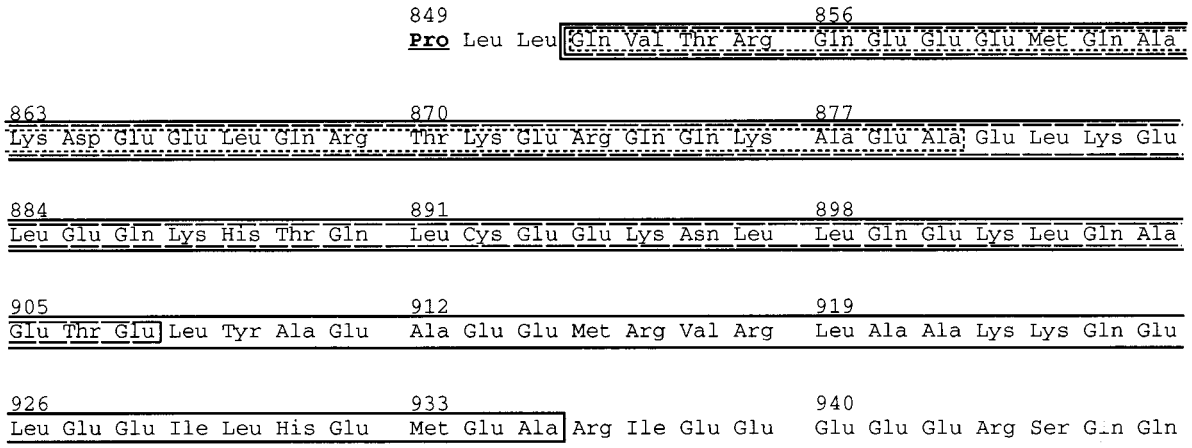

FIGURE 2: Amino acid sequence of smooth muscle myosin heavy chain at S2 near the invariant proline. The invariant proline at the head−rod junction is shown in boldface type. The dedelted amino acid residues for ΔA, ΔAB, and ΔABC are boxed by dotted lines, broken lines, and solid lines, respectively. The heptapeptide repeat in the myosin rod is indicated.

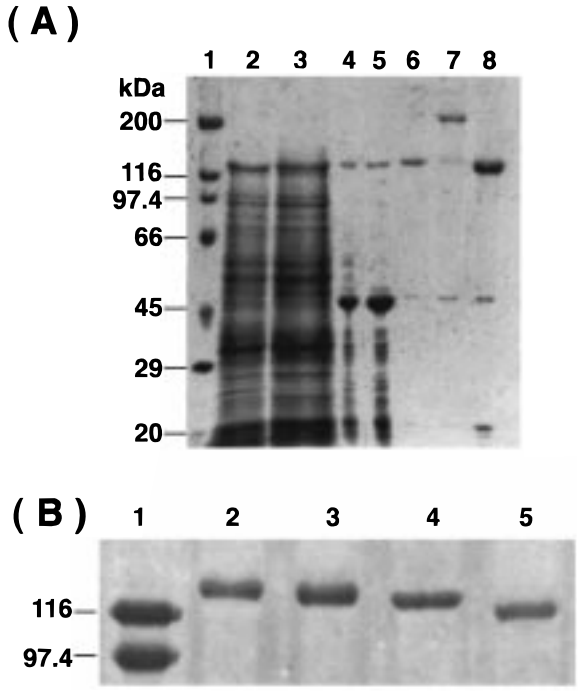

FIGURE 3: Purification of the smooth muscle myosin mutants. (A) SDS−PAGE of each purification step of the wild-type truncated myosin. lane 1, molecular mass standards; lane 2, total cell homogenate; lane 3, supernatant of the cell homogenate; lane 4, pellets after centrifugation in the presence of actin; lane 5, pellets after centrifugation in the presence of ATP; lane 6, supernatant after centrifugation in the presence of ATP; lane 7, pellets after dialysis at low ionic strength; lane 8, purified wild-type truncated myosin. (B) SDS PAGE of the purified smooth muscle myosin mutants. Lane 1, molecular mass standards; lane 2, ΔA-myosin; lane 3,Δ AB-myosin; lane 4, ΔABC-myosin; lane 5, wild-type truncated nyosin.

phosphorylation. While the nature of the interaction between the two heads that inhibits the motor activity is still obscure, previous results have suggested that the light chain binding domain of S1, or the N-terminal region of S2, may be critical for such an interaction because the chimeric myosin which is composed of the skeletal globular motor domain, the smooth muscle light chain binding domain, and short S2 shows complete phosphorylation dependence for both its actin-activated ATPase activity and its actin translocating activity (*18*). The present results provide clear evidence that the specific sequence at the head−rod junction of smooth muscle myosin is not critical for the phosphorylation-mediated regulation of smooth muscle myosin motor activity. This strongly suggests that the inhibition of myosin motor activity in the dephosphorylated form is achieved solely by the interaction between the light chain binding domain of the two heads. The present results along with the previous findings suggests that the S2 portion of the molecule is important to hold the two heads close to each other, thus enabling the light chain bound domains to interact with each other.

Recently, Trybus et al. (*19*) suggested that there is a specificity in the rod structure which is required for phosphorylation-dependent regulation. They introduced the leucine zipper in smooth muscle rod after two heptads and seven heptads of rod sequence, respectively. These smooth muscle myosins with the leucine zipper in S2 showed only partial phosphorylation-dependent regulation. Based on this result, they suggested that a specific sequence of the rod mediates an interaction between the heads that is essential to achieve an inhibited state of smooth muscle myosin and if the wrong sequences are placed at a certain distance down the rod then the regulation is hampered. The amino acid residues at a certain distance down the rod of the three S2 deletion mutants produced in this study are different from each other. This suggests that specific residues at S2 are not required for regulation. Although the discrepancy between their results and the present results is unclear, some possibilities may account for the difference in the results. As a leucine zipper is a strong coiled-coil motif in which only 30 residues are sufficient for dimerization (*39*), the introduction of a leucine zipper in S2 may reduce the flexibility of the coiled-coil, thus fixing the configuration of the 2 heads, making them less accessible to each other. Another possibility is that the introduction of a 32 residue leucine zipper may disrupt the phase of 7 and/or 28 residue repeat pattern found in myosin rod and this may affect the regulation. In both cases, an exact amino acid sequence in S2 would not be critical for the regulation of smooth muscle myosin. Interestingly, when skeletal S2 was attached to the smooth muscle myosin head, the ATPase activity was well regulated although the motility data were not shown (*19*). Approximately 35% of the residues in the skeletal and smooth muscle myosin S2 regions

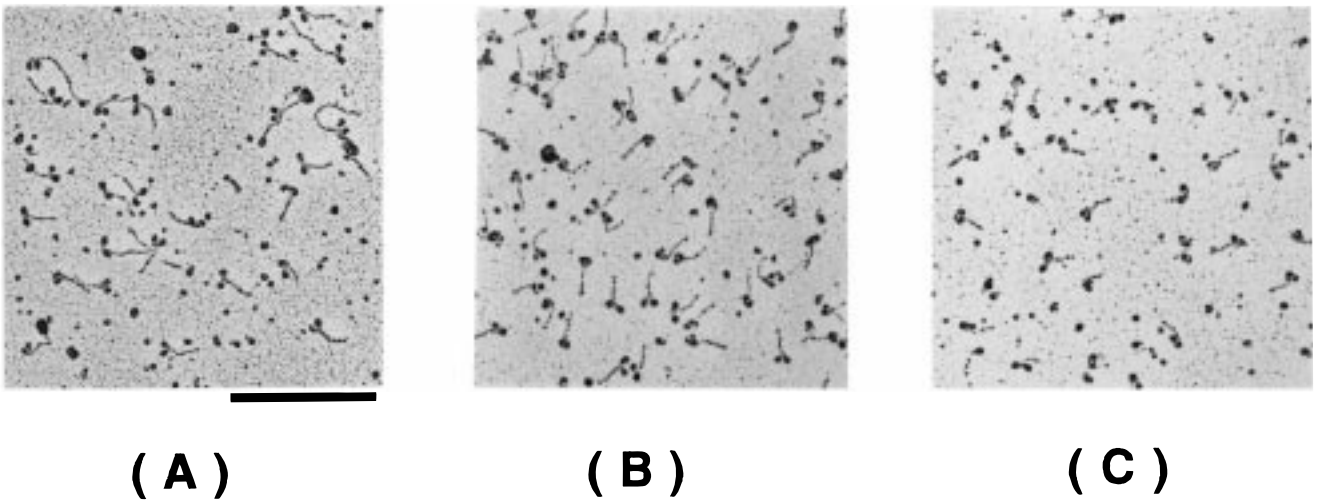

FIGURE 4: Electron micrographs of the smooth muscle myosin mutants. The rotary-shadowed images of the smooth muscle myosin mutants clearly show the two-headed structure of the truncated myosin mutants [(A) ΔA-myosin; (B) ΔAB-myosin; (C) ΔABC-myosin]. Magnification is 64000 × bar = 0.3 μm.

Table 1: Actin-Activated ATPase Activity of Smooth Muscle Myosin Mutants in Which S2 Amino Acid Residues Are Deleted to Various Extents[a]

	V_{max} (s^{-1})		
myosin	phosphorylated	dephosphorylated	regulation (*x*-fold)
HMM	0.86	0.060	14.3
Wild	0.77	0.050	15.4
ΔA	0.55	0.043	12.3
ΔAB	0.76	0.064	11.7
ΔABC	0.86	0.070	12.2

[a] Actin-activated ATPase activity was measured at 25 °C in 0.1 mg/mL myosin mutant, 0.3 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 myosin light chain kinase, and 10 μg/mL calmodulin were added, whereas 1 mM EGTA was added for the dephosphorylated one. Results are means of five independent experiments. A computed nonlinear least-squares curve-fitting program was used to estimate the maximum actin-activated ATPase activity (V_{max}): $V = V_{max}/(1 + K_a/[\text{actin}])$. Mg^{2+}-ATPase activity in the absence of actin was subtracted. Degree of regulation is the ratio of the phosphorylated to dephosphorylated actin-activated ATPase.

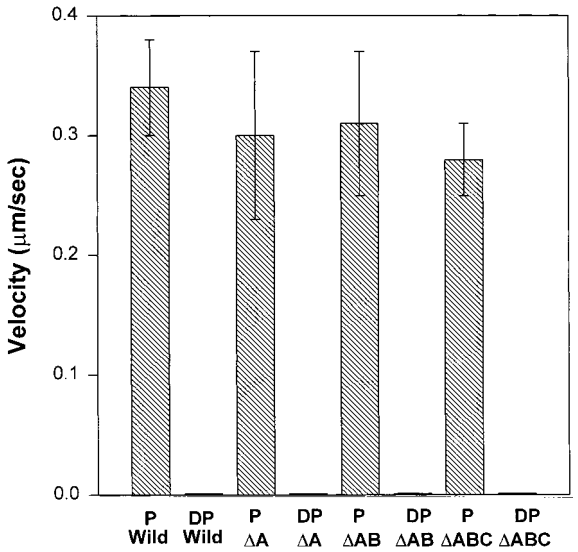

FIGURE 5: Sliding velocity of actin filaments on the smooth muscle myosin mutants. Actin movement was observed in 30 mM KCl, 5 mM $MgCl_2$, 25 mM imidazole, 1 mM EGTA, 1% 2-mercaptoethanol, 0.5% methylcellulose, 4.5 mg/mL glucose, 216 μg/mL glucose oxidase, 36 μg/mL catalase, 2 mM ATP, pH 7.5 at 25 °C. All values are mean velocities ± SD.

are identical, and Trybus et al. (*19*) concluded that the conserved residues between the two myosins at their S2 are responsible for regulation. However, together with the present results, it can be concluded that a particular sequence at S2 is not important for smooth muscle regulation.

Recently, it was proposed by a modeling study that a region near the N-terminus of the scallop regulatory light chain lies close to heavy chain residues 837−841 at the C-terminal side of the invariant proline (*40*), suggesting contact between each regulatory light chain and its heavy chain partner in the rod portion. Within this region, it is known that a positively charged residue at position 841 (corresponding to Arg855 in gizzard heavy chain) is a common feature of all regulated myosins (*41*, *42*) and Arg841 in the scallop heavy chain lies close to the acidic residues in the regulatory light chain (*40*) which is conserved in the smooth muscle myosin regulatory light chain. In the present study, the deletion of the segments (Leu851−Ala879, Leu851−Glu907 or Leu851−Ala935) containing these residues did not diminish the phosphorylation-dependent regulation of smooth muscle myosin motor function. The results suggest that segment Leu851−Arg855 in the smooth muscle heavy chain (corresponding to Leu837−Arg841 of the scallop heavy chain) is not critical for regulation, and may not interact with the regulatory light chain.

A number of studies have addressed the possibility that a helix−coil transition in S2 may be involved in the force production of the cross-bridge (*43*). Recent studies have questioned this hypothesis because S1, or even the motor domain alone (*44*), can generate force, indicating that S2 does not play a role in motor function. The present results are consistent with this notion, since deletion of the S2 residues showed no effect on the in vitro actin translocating activity of myosin, although it cannot exclude the possibility that the S2 region might affect loaded contraction.

The information obtained in this study along with the earlier findings (*15*−*18*) suggest that the interaction between the two heads of smooth muscle myosin at the regulatory

light chain binding region of the molecule is critical to account for the phosphorylation-mediated regulation of smooth muscle myosin. Further study at a molecular level is required to clarify the more detailed nature of the molecular mechanism of regulation.

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