Registration of the Rod Is Not Critical for the Phosphorylation-Dependent Regulation of Smooth Muscle Myosin†

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ABSTRACT: A recent report has suggested that the interaction between the head and the rod region of smooth muscle myosin at S2 is important for the phosphorylation-mediated regulation of myosin motor activity [Trybus, K. M., Freyzon, Y., Faust, L. Z., and Sweeney, H. L. (1997) *Proc. Natl. Acad. Sci. U.S.A. 74*, 48−52]. To investigate whether specific amino acid residues at S2 or whether the registration of the 7-residue/28-residue repeat appearing in the α-helical coiled-coil structure of the rod are critical for such an interaction, two smooth muscle myosin mutants were constructed in which the N-terminal sequences of S2 were deleted to various extents. One mutant contained a deletion of 71 residues at the position immediately C-terminal to the invariant proline (Pro849) linking the S1 domain directly to the downstream sequence of the rod, while in another mutant, 53 residues were deleted at a position 56 residues downstream of Pro849. Despite these alterations which change the registration of both the 28-residue repeat and the 7-residue repeat found in myosin rod sequence, both myosin mutants showed a stable double-headed structure by 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 two mutant myosins was the same as the wild-type recombinant myosin. Furthermore, the head configuration critical for myosin filament formation (extended or folded) was unchanged in either mutant. These results indicate that neither the specific amino acid residues nor the registration of the amino acid repeat in S2 is critical for the head configuration. These results indicate that neither a specific amino acid sequence at the head−rod junction nor the rod sequence registration is critical for the regulation of smooth muscle myosin.

Vertebrate smooth muscle and nonmuscle myosin function is regulated by the phosphorylation of the regulatory light chain at Ser19, catalyzed by Ca^{2+}-calmodulin-dependent myosin light chain kinase (*1*−*5*). Phosphorylation of the RLC[1] at Ser19 regulates the myosin function in two respects. First, phosphorylation increases the actin-dependent ATPase activity, thus activating the motor activity of myosin (*1*−*5*). Second, phosphorylation alters the conformation of myosin, by stabilizing myosin thick filament formation (*1*−*5*). The regulatory light chain is associated at the C-terminal end of S1, and located distal to the ATP binding site and actin binding site (*6*). Of interest is how the phosphorylation at Ser19 affects smooth muscle myosin's motor activity and filament formation. The phosphate moiety at the side chain of Ser19 is quite distant from both the catalytic and actin binding sites, which are both localized toward the top of the myosin head (*6*, *7*). This suggests that phosphorylation activates the motor activity indirectly, presumably via light chain−heavy chain intersubunit communication (*8*).

Recent evidence suggests that the interaction between the two heads of myosin is critical for phosphorylation-mediated regulation. Originally this was suggested by the finding that the actomyosin-activated ATPase activity of HMM-but not S1-containing intact regulatory light chain is regulated by phosphorylation (*9*). These findings are supported by recent reports that the motor activity of single headed myosin (*10*) and single-headed long S1 (*11*, *12*) is not regulated by phosphorylation whereas double-headed short HMM is so regulated (*11*, *12*). A chimeric myosin consisting of the skeletal globular motor domain and the smooth muscle light chain associated domain was found to be regulated by phosphorylation of the regulatory light chain, demonstrating that it is the light chain associated domain and not the motor domain that is responsible for the interhead interaction (*13*). It was also reported that when the skeletal light chain binding domain is connected to the smooth muscle myosin motor domain, in vitro motility is still partially regulated (*14*), and it is proposed that the "converter" domain is also involved in regulation. On the other hand, Trybus et al. (*15*) recently

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminothyl 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; RLC, regulatory light chain of myosin; DTT, dithiothreitol.

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showed that the S2 portion of the myosin molecule plays an important role in the phosphorylation-dependent regulation of smooth muscle myosin. It was found that the introduction of a leucine-zipper motif into the S2 portion of the myosin molecule resulted in the production of unregulated myosin, suggesting that the S2 portion may play an important role in the regulation of smooth muscle myosin motor function. Based on this result, it was proposed that the head may interact with specific residues at S2, and that this interaction is important for the regulation of myosin motor function. It is known that the myosin rod shows a unique 28-residue repeat that is critical for α-helical coiled-coil structure of the myosin tail (*16*, *17*). The introduction of a leucine zipper into the rod is expected to change the registration of this 28 amino acid repeat structure. A question arises whether the unique amino acid sequence at the head−rod junction in smooth muscle myosin or the 28 amino acid residue/7 amino acid residue repeat located in the rod sequence is critical for the phosphorylation-dependent regulation of motor activity and the head configuration that is critical for thick filament formation (*1*−*5*).

In the present study, we produced smooth muscle myosin mutants in which the amino acid residues at the head−rod junction in S2 were deleted to various extents, thus altering the registration of the amino acid repeat structure in the rod. These mutant myosins were expressed in Sf9 cells, and then isolated and characterized in order to investigate whether this region of the molecule plays a critical role in the regulation of myosin function.

MATERIALS AND METHODS

Materials. Restriction enzymes and modifying enzymes were purchased from New England Biolab (Beverly, MA). Smooth muscle myosin and smooth muscle myosin light chain kinase were prepared from frozen turkey gizzards as described (*18*, *19*). Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (*20*). Recombinant calmodulin from *Xenopus* oocytes (*21*) was expressed in *E. coli* and prepared as described (*22*).

Expression of the Recombinant Smooth Muscle Myosin Mutants. Smooth muscle myosin heavy chain cDNA was obtained from chicken gizzard as described previously (*11*). 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 (*8*, *23*). *Nde*I sites were introduced at the 905th and 958th codons, or 851st and 922nd 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 the amino acid residues Thr906−Met958 (ΔC25/28D) and Gln852−Lys922 (ΔAB15/28C), respectively. The truncated cDNA was purified by agarose gel electrophoresis and then self-ligated. After confirmation of the mutation by direct sequencing analysis (*24*), 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 *Nhe*I 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 direct sequencing analysis. The baculovirus transfer vectors of smooth muscle light chains were produced as described (*12*). Recombinant baculoviruses for the heavy chain and the light chains were produced according to the protocols described by O'Rielly et al. (*25*). The recombinant virus expressing myosin heavy chain (Met1−Ser1110) (*11*) was used to prepare 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 (*12*).

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, 1 mM EGTA, 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 (*18*). SDS−PAGE was carried out on 7.5−20% polyacrylamide gradient slab gels by using a discontinuous buffer system of Laemmli (*26*). Molecular mass markers used were smooth muscle myosin heavy chain (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (27 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 (*12*). 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 (*27*). The specimen was visualized by the rotary shadowing technique according to Mabuchi (*28*) with an electron microscope (Phillips Electronic Instruments, Mahwah, NJ; Model EM300) at 60 kV.

RESULTS

Expression and purification of smooth muscle myosin mutants. α-helical coiled-coil proteins show a characteristic seven residue repeat pattern where the hydrophobic residues in the first and fifth positions form a hydrophobic core of the coiled-coil (*29*). The myosin rod contains this characteristic feature, and in addition, it shows a 28-residue periodicity (four heptapeptide repeat) with alternating bands of positive and negatively charged residues (*16*, *30*−*32*). In the smooth muscle myosin rod, a 28-residue repeat starts with Pro849 at position 15 of the 28-residue repeat (*17*). In the present study, we produced 2 mutant myosins both of which altered its 28-residue/7-residue registration in order to investigate whether the periodicity of the rod amino acid sequence is critical for the phosphorylation-mediated regulation of smooth muscle myosin motor function as well as

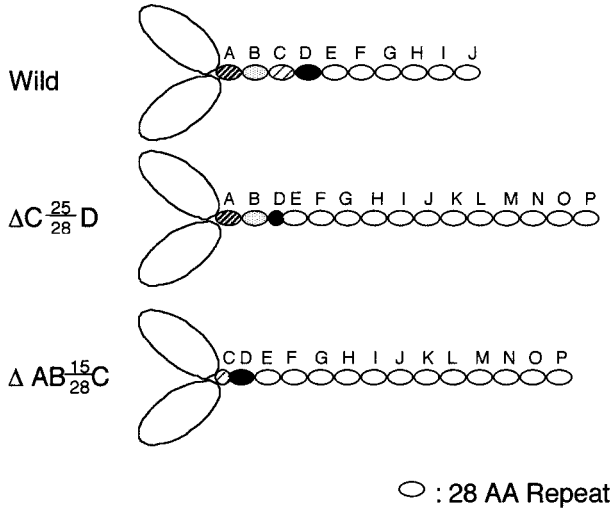

FIGURE 1: Schematic drawing of the smooth muscle myosin mutants in which the amino acid residues at S2 are deleted.

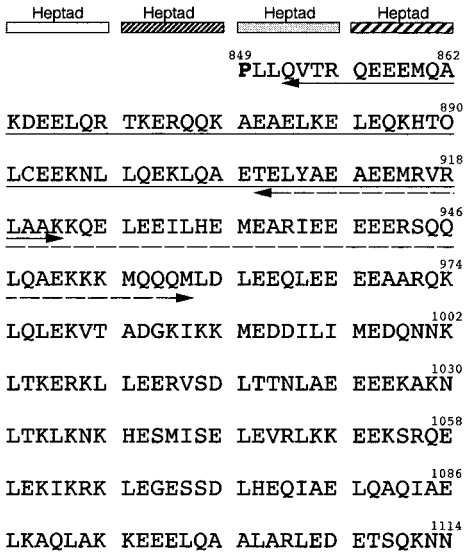

FIGURE 2: Amino acid sequence of chicken gizzard myosin heavy chain at S2. The invariant proline at the head−rod junction is shown in boldface type. The deleted amino acid residues for ΔAB15/28C and ΔC25/28D are underlined by solid and dashed lines, respectively. The 28-residue repeat is represented by each line. The single-letter code is used to represent amino acid residues.

filament formation. In one mutant myosin (ΔAB15/28C), the 71 amino acid residues next to the invariant proline (Pro849) were deleted. The resulting myosin lacks the original 71 amino acid residues at the head−rod junction, and, moreover, this alters the phase of the coiled-coil structure from the beginning of the rod structure. In the other one, 53 residues after the two, 28-residue repeats were deleted (ΔC25/28D). In this mutant, the registration of the rod sequence is altered at the middle of S2 (Figure 1). Smooth muscle myosin heavy chain cDNA encoding Met1−Val1298 was made by introducing a stop at codon 1299. The construct is expected to express a myosin fragment containing 16, 28-residue repeats, which is sufficient to produce a stable 2-headed structure critical for the regulation of smooth muscle myosin (*11*, *12*). This cDNA construct was used as a template for site-directed mutagenesis to produce the S2 deletion mutants. The deleted amino acid residues and 28-residue registration in the S2 portion of the molecule are shown in Figure 2.

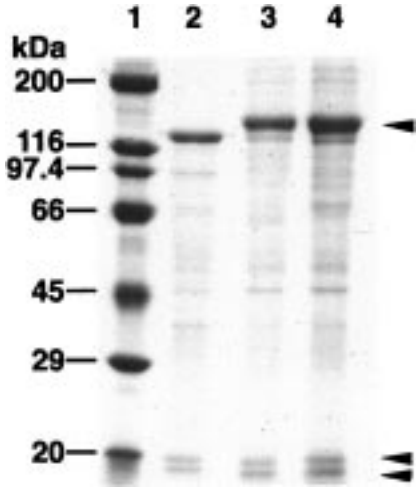

FIGURE 3: SDS−PAGE of the purified smooth muscle myosin mutants. Lane 1, molecular mass standards; lane 2, wild-type truncated myosin; lane 3, Δ AB15/28C myosin; lane 4, Δ C25/28D myosin. The arrows show the expressed myosin heavy chain, RLC, and essential light chain, respectively.

Table 1: Actin-Activated ATPase Activity of Smooth Muscle Myosin Mutants in Which the Rod Registration Is Altered[a]

	phosphorylated		dephosphorylated		
myosin	V_{max} (s^{-1})	K_a (μM)	V_{max} (s^{-1})	Ka (μM)	regulation
wild	0.77	83	0.050	101	15.4-fold
ΔAB15/28C	0.57	79	0.057	107	10.0-fold
ΔC25/28D	0.63	83	0.060	115	10.5-fold

[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, pH 7.5, 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 dephosphorylated myosin. 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}): $\boldsymbol{V} = V_{max}/(1 + K_a/[\text{actin}])$. The ATPase activity in the absense of actin was subtracted. The degree of regulation is the ratio of the phosphorylated to dephosphorylated actin-activated ATPase.

Figure 3 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 mass of the heavy chain of the mutants was as expected from the cDNA constructs. No endogenous myosin, i.e., Sf9 myosin, having a 200 kDa molecular mass was observed, indicating that the obtained preparations were free from endogenous myosin. This was further supported by observations using electron microscopy. As shown in Figure 4, both ΔC25/28D and ΔAB15/28C myosins showed a double-headed structure. Virtually no full-length myosin molecules were observed, judged from the tail length of myosin, suggesting that there was practically no contamination of endogenous myosin.

V_{max} of the Actin-Activated ATPase Activity of the 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 as described in Table 1. For both myosin mutants (ΔC25/28D and ΔAB15/28D), the V_{max} of the actin-activated

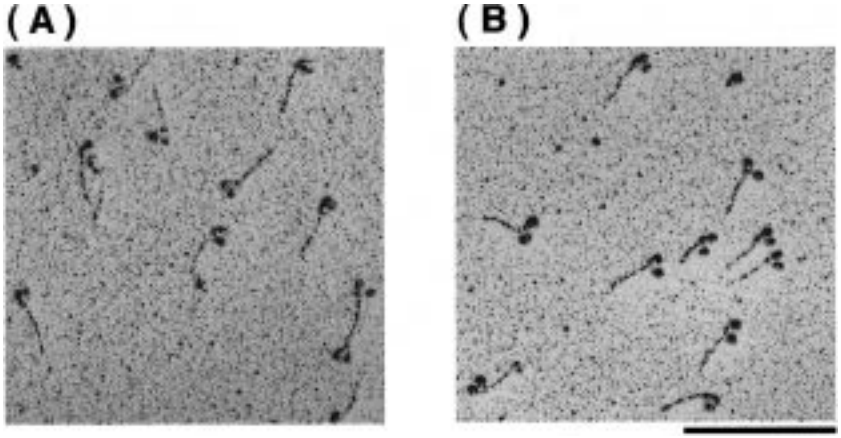

FIGURE 4: Electron micrographs of the smooth muscle myosin mutants. The rotary-shadowed images of the smooth muscle myosin mutants clearly show the two-headed structure of the truncated myosin mutants [(A) ΔAB15/28C myosin; (B) ΔC25/28D myosin]. Magnification is 100000*x*; bar = 0.2 μm.

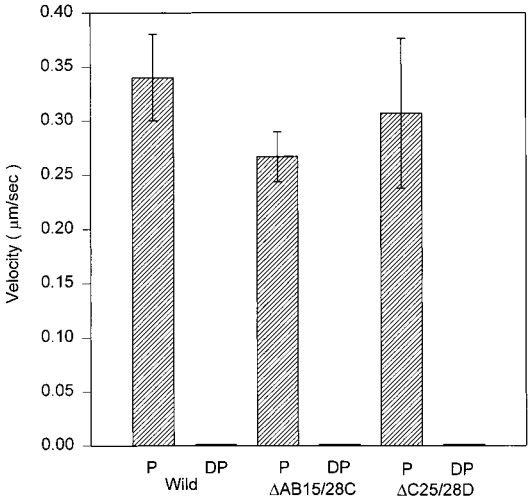

FIGURE 5: Sliding velocity of actin filaments on the smooth muscle myosin mutants. Actin movement was observed in 30 mM KCl, 5 mM $MgCl_2$, 25 mM imidazole, 1 mM EGTA, 1% 2-mercaptoethanol, 0.5% methylcellulose, 4.5 mg/mL glucose, 216 mg/mL glucose oxidase, 36 mg/mL catalase, 2 mM ATP, pH 7.5 at 25 °C.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

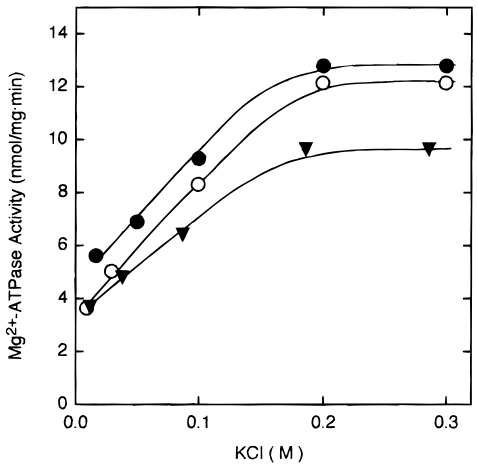

FIGURE 6: KCl dependence of Mg^{2+}-ATPase activity of myosin mutants. Mg^{2+}-ATPase activity was measured in 0.01 mg/mL myosin, 1 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.5), and various concentrations of KCl at 25 °C. (○) Wild type; (●) ΔAB15/28C; (▼) ΔC25/28D.

ATPase activity was significantly enhanced by phosphorylation of the regulatory light chain (Table 1). The apparent dissociation constant for actin of the mutant myosins (K_a) was similar to that of the wild-type truncated myosin fragment, which contained intact S2 amino acid sequence. This result clearly indicates that neither the specific amino acid sequence at the head−rod junction (or S2) nor the registration of a 28-residue/7-residue repeat in the rod is critical for light chain phosphorylation-dependent regulation of the actin-activated ATPase activity of smooth muscle myosin.

Actin-Translocating Activity of the Smooth Muscle Myosin Mutants. To evaluate more directly the motor activity of the various myosin mutants, the actin-translocating velocity of the deletion mutants and 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 actin-translocating activity of the smooth muscle myosin mutants in which S2 is deleted to different extents (thus altering the registration of the sequence repeat) was completely regulated by light chain phosphorylation, and no motility activity was observed for the dephosphorylated forms. The actin-translocating velocity of the two phosphorylated myosins was indistinguishable from that of the wild-type recombinant myosin fragment. These results were consistent with the actin-activated ATPase activity and indicated that neither the specific amino acid sequence at the head−rod junction nor the registration of the sequence repeat is critical for the regulation of smooth muscle myosin motor activity.

Conformational Transition of the Smooth Muscle Myosin Mutants. Smooth muscle myosin or HMM forms a folded conformation in low ionic strength (*33*−*35*), which is characterized by a low Mg^{2+}-ATPase activity of myosin (*36*). As shown in Figure 6, the Mg^{2+}-ATPase activity of the dephosphorylated form decreased at low ionic strength for both mutants. This property was similar to that of the wild-type myosin fragment (Figure 6) as well as naturally isolated HMM (*37*), suggesting that both S2 deletion mutants undergo a conformational transition at low ionic strength. To detect the conformational transition more directly, the mutant myosin fragments were subjected to electron microscopy. Smooth muscle myosin molecules show two distinct orientations of the heads in terms of the long rod axis. In the extended form, the heads extend away from the tail, whereas the heads fold back toward the tail in the folded conformation. The difference between the two conformations can be

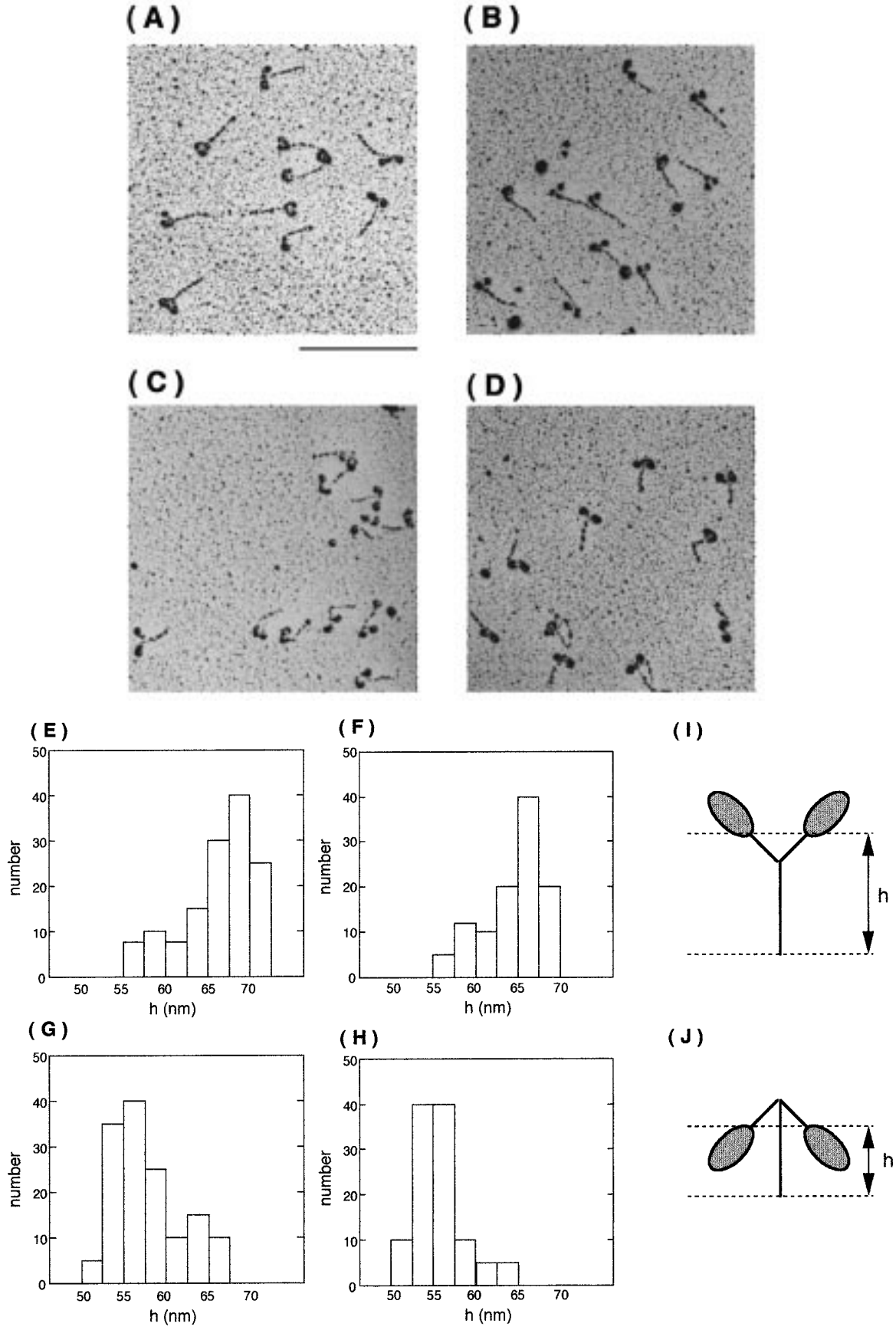

FIGURE 7: Electron micrographs of the myosin mutants in which the rod registration is altered. Rotary-shadowed images of the smooth muscle myosin mutants in 0.4 M KCl (A, B) and 0.01 M KCl (C, D). ΔAB15/28C (B, D) and ΔC25/28D (A, C). The distributions of the height of the globule/α-helix junction (*h*) of ΔAB15/28C in 0.4 M KCl (F) and 0.01 M KCl (H) and ΔC25/28D in 0.4 M KCl (E) and 0.01 M KCl (G) are also shown. The height was measured according to the schematic drawing for the extended (I) and the folded (J) forms. Magnification is 100000*x*; bar = 0.2 μm.

statistically analyzed by measuring the length between the globular head/α-helix junction from the C-terminal end of the tail. The mean *h* values (see Figure 7) of ΔAB15/28C myosin and ΔC25/28D myosin at 0.4 M NaCl were 64.5 ± 3.5 and 66.3 ± 4.2 nm, respectively, while the values at 10 mM NaCl were 55.7 ± 2.9 nm and 57.7 ± 3.9 nm, respectively. The results clearly indicate that both mutants undergo the conformational transition at low ionic strength like native smooth muscle myosin.

DISCUSSION

Two smooth muscle myosin mutants were produced in which the registration of the 28-residue/7-residue repeat found in the coiled-coil structure of myosin rod was altered. The obtained results show that the registration of the 28-residue/7-residue repeat in the rod is not critical for the regulation of smooth muscle myosin motor activity since the isolated mutant myosins showed complete phosphorylation dependence for their actin-activated ATPase and actin-translocating activity. Quite recently, it was shown (*38*) that deletion of the amino acid residues in S2 does not influence phosphorylation-dependent regulation, suggesting that the specific sequence in the S2 region is not required for regulation. However, integral multiples of the 28-residue repeat are deleted from S2 amino acid residues in the earlier report, and thus the registration of the repeat structure in the rod was unaltered. It was reported previously (*15*) that a specific sequence in S2 is critical for interaction with myosin heads that is involved in phosphorylation-dependent regulation. The conclusion was made based upon the results that the introduction of a leucine zipper in the smooth muscle rod after 2 heptads or 7 heptads of the rod sequence resulted in only partial phosphorylation dependent regulation of myosin. While the reason for the discrepancy between the two earlier reports is unclear, it would be possible that the introduction of a leucine zipper changes the registration of the rod structure, thus disrupting the regulation. The present study sought to test this important possibility. The present results agree with the earlier report (*38*) and furthermore show that not only the specific sequence in S2 but also the registration of the repeat structure is unnecessary for the phosphorylation-dependent regulation of motor activity.

The apparent discrepancy between the present results and those of Trybus et al. (*15*) is unclear. One possibility is that the introduction of a leucine zipper may alter the accessibility of the heads to each other. 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 two heads and making them less accessible to each other. It was shown previously that single-headed myosin (*10*) and long S1 (Met1−Gln957) (*11*, *12*) 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 (*11*) as well as in vitro motility activity (*12*). These results indicate that the interaction between the two heads is critical for the inhibition of smooth muscle motor activity and the regulatory light chain phosphorylation releases this inhibition.

Recently, it was shown that chimeric myosin, which is composed of the skeletal globular motor domain and the smooth muscle light chain binding domain and short S2, shows complete phosphorylation dependence for both its actin-activated ATPase activity and actin-translocating activity (*13*). Therefore, the light chain binding domain of S1 may be critical for such an interaction between the two heads. The present results provide clear evidence that the specific sequence at the head−rod junction and the rod registration of smooth muscle myosin are 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 by the interaction between the light chain binding domain of the two heads. Quite recently, it was reported that the "converter" domain of myosin might influence the regulation in addition to the light chain binding domain (*14*). In that study, the skeletal myosin light chain binding domain and the skeletal myosin light chain binding domain + skeletal myosin converter domain were introduced into the smooth muscle myosin heavy chain sequence. It was found that the actin-translocating velocity of these chimeric myosins becomes unregulated and 56% and 86% of the maximum velocity (observed for the phosphorylated myosin) were observed for the dephosphorylated form of these mutants, respectively. It was also shown previously that the complete deletion of the actin binding flexible loop of myosin disrupted the regulation (*40*), although the partial deletion of this loop left the regulation unaffected (M. Ikebe, X. Li, and R. Ikebe, unpublished observation). These results suggest that disruption of the structure of the motor domain can influence the interaction between the heads, thus diminishing the regulation, although it is unclear whether this is due to the disruption of a direct interaction between the motor domains in two heads or the disruption of the configuration of the light chain binding domain between the two heads. Based upon these findings, it is plausible that the proper configuration of the two heads is important to achieve the inter-head interaction that is critical for stabilizing the inhibited form of myosin. The function of the rod in terms of regulation would be to hold the two heads in the proper position to achieve proper inter-head interaction.

It is known that smooth muscle myosin shows two distinct head orientations with respect to the rod (*33*−*35*). In one of the configurations, the head bends back toward S2 (folded form), and the myosin head seems to interact with the S2 portion of the molecule. Since phosphorylation at Ser19 of the RLC inhibits the formation of the folded conformation, it has been assumed that there may be a charge interaction between the head domain and S2. It was shown that Arg16 and Arg13 of RLC are critical for the formation of the folded structure of smooth muscle myosin (*8*, *41*), suggesting that these basic residues interact with the acidic residues in the rod. Present results, however, indicate that the residues in the S2 portion of the molecule do not play a role in such an interaction with the basic residues of RLC. It would be plausible that the formation of the folded conformation is induced by the inter-head interaction, presumably between the two RLC on each head.

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