Requirement of the two-headed structure for the phosphorylation dependent regulation of smooth muscle myosin

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Abstract It is known for smooth muscle myosin that while acto-HMM ATPase activity is regulated by phosphorylation, acto-S-1 ATPase activity is not regulated. To clarify the heavy chain structure required for the regulation, smooth muscle myosin containing 7 different lengths of the S-2 portion were expressed in Sf9 insect cells using Baculovirus expression system. Myosin containing longer than 991 residues of heavy chain formed a stable two-headed structure while myosin with shorter than 944 residues of heavy chain formed a single-headed structure, indicating that the residues Gln^{945}–Asp^{991} are critical for the fomation of the two-headed structure. The actin activated ATPase activity of myosin mutants having a two-headed structure was activated by phosphorylation while that of myosin mutants that failed to form the two-headed structure was completely independent of phosphorylation. These results suggest that the two-headed structure is critical for the phosphorylation-dependent regulation.

Key words: Myosin; Smooth muscle; Phosphorylation; Mg^{2+}-ATPase activity; Baculovirus expression

1. Introduction

It is known that the motor activity of vertebrate smooth muscle and non-muscle acto myosin is activated by 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) [1–3]. The activation of actomyosin requires the phosphorylation of rather specific sites, i.e. Ser^{-19} and Thr^{-18} [4–7] and the phosphorylation at Ser^1/Ser^2 and Thr^{-9} catalyzed by protein kinase C [8,9] failed to activate actomyosin motor activity [8–10]. The question is how the phosphorylation at Ser^{19}/Thr^{18} of the 20,000 Da light chain can activate actomyosin motor activity. It is unlikely that the phosphate moiety interacts directly with the effector sites, i.e. the ATP binding site and/or actin binding site, because the light chain is localized at the head–rod junction [11–12] which is quite distal to the effector sites located towards the top of the myosin head [11]. It is plausible that the phosphorylation changes the conformation of the 20,000 Da light chain which is transmitted to the myosin heavy chain thus changing the motor activity. This view is supported by recent findings that the C-terminal domain of the 20,000 Da light chain is important for regulation [12–14] and

the elimination of the C-terminal residues of the light chain abolishes phosphorylation-dependent activation of actomyosin motor [13,14], suggesting that the phosphorylation induced conformational change is transmitted to heavy chain via the C-terminal domain of the light chain. While the light-chain binding site is within the S-1 domain of myosin the phosphorylation-dependent regulation requires the S-2 domain since S-1 containing the intact light chains is not regulated by phosphorylation whereas HMM containing the S-2 domain is regulated [15]. The importance of the S-2 region for regulation is supported by a series of findings [15–20] that the conformation at the S-1-S-2 junction of smooth muscle myosin is altered by light chain phosphorylation. To further elucidate the function of the S-2 domain on the regulatory mechanism, we produced various myosin mutants which contain various lengths of the S-2 portion using a Baculovirus expression system.

2. Materials and methods

2.1. Materials
Smooth muscle myosin was prepared from frozen turkey gizzards as described previously [21]. Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt [22]. Myosin light chain kinase was prepared from frozen turkey gizzards [23]. Calmodulin was prepared from bull testes [24]. Myosin light chains were prepared from smooth muscle myosin according to Hathaway and Haebele [25] with modification [26].

2.2. Construction of myosin heavy chain expression vector and expression of the recombinant myosin heavy chain
The cDNA containing entire open reading frame of myosin heavy chain was obtained from chicken gizzard λzap cDNA library using oligonucleotide probes designed according to the published sequence [27]. The screening was carried out as described previously [7] and the sequence was confirmed by dideoxynucleotide termination method [28] using Sequenase 2.0 (US Biochemical).

pBluescript SKII(−) containing myosin heavy chain cDNA (−44–6,016) was excised automatically from λ phage on M13 helper phage infection. Using a transformer Site Directed Mutagenesis Kit (Clontech Inc.), endogenous unique *Spe*I site was deleted without changing amino acid residues and an additional *Spe*I site was created at the 19th position (Fig. 1). Using the same site directed mutagenesis strategy, a stop codon was introduced at 2,551, 2,617, 2,833, 2,974, 3,331, or 3,439, thus producing the truncated heavy chains of Met^1–Leu^{850}, Met^1–Glu^{872}, Met^1–Ser^{944}, Met^1–Gln^{957}, Met^1–Asp^{991}, Met^1–Ser^{1110}, or Met^1–Glu^{1153}, respectively. The myosin heavy chain cDNA in SKII(−) was digested with *Spe*I and subcloned into pBlueBacM Baculovirus transfer vector using unique *Nhe*I site in the multicloning site which is localized downstream of the polyhydrin promotor (Fig. 1). The recombinant smooth muscle myosin heavy chains were expressed in the insect cells by the protocol described [29].

2.3. Other biochemical procedures
SDS PAGE was carried out on 7.5% polyacrylamide slab gels by using the discontinuous buffer system of Laemmli [30]. Molecular mass markers used were smooth muscle myosin heavy chain (200 kDa),

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β-galactosidase (116 kDa), phosphorylase a (97.4 kDa) and bovine serum albumine (66 kDa). Western blotting was performed as described previously [20] with modification [31]. ATPase activity of the recombinant myosin fragments was measured as described previously [15]. The protein concentration of myosin was estimated from densitometric analysis of the gel (USB Sciscan 5000, US Biochemical, Cleveland, OH) using natural myosin as a standard.

2.4. Preparation of the recombinant myosin heavy chain mutants

Sf-9 cells infected with the recombinant virus were harvested after 72 h infection and lysed with 5 vol. of the buffer containing 400 mM KCl, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.2 mM EGTA, 10 mM ATP, 5 mM DTT, 2 mM PMSF, 2 mM Nα-p-Tosyl-L-arginine methylester, 0.2 mM Nα-p-Tosyl-L-phenylalanine chloromethyl ketone, 0.2 mM Nα-p-Tosyl-L-lysine chloromethyl ketone, 0.01 mg/ml of leupeptin, 1 mg/ml of trypsin inhibitor, 0.5% Triton X-100, 1% NP-40, 1 M monosodium glutamate, 100 μg/ml of 20,000 Da light chain and 100 mg/ml of 17,000 Da light chain with sonication. The cell lysate was subjected to 30–35% ammonium sulfate fractionation. The resulting precipitates were collected and dissolved in 3 ml of 400 mM KCl, 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.2 mM PMSF and 0.01 mg/ml leupeptin. The solution was clarified by centrifugation and the resulting

clear supernatant was applied to a column (2.5 × 20 cm) of G-25 which had been equilibrated with 400 mM KCl, 50 mM Tris-HCl (pH 7.5), 5 mM DTT and 0.2 mM PMSF. Proteins were eluted with the same buffer and the first peak was collected. F-actin (final concentration of 1 mg/ml) was added to this fraction and the recombinant myosin was coprecipitated with centrifugation (100,000 × g for 30 min). The pellet was washed with the same buffer then washed with 15 mM KCl, 30 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.2 mM PMSF and 10 mM MgCl₂. The pellet was dissolved with the buffer containing 0.1 mM ATP then centrifuged at 100,000 × g for 30 min. The supernatant containing the recombinant myosin was subjected to the assay.

3. Results and discussion

Smooth muscle myosin heavy chains with various tail lengths were expressed in the insect cell line Sf9. Fig. 2A shows SDS PAGE of Sf9 cell total homogenate expressing various truncated myosin heavy chain mutants. Apparent molecular mass of the seven truncated mutants were 134 kDa, 128 kDa, 114 kDa, 110kDa, 108 kDa, 100 kDa and 97 kDa which were
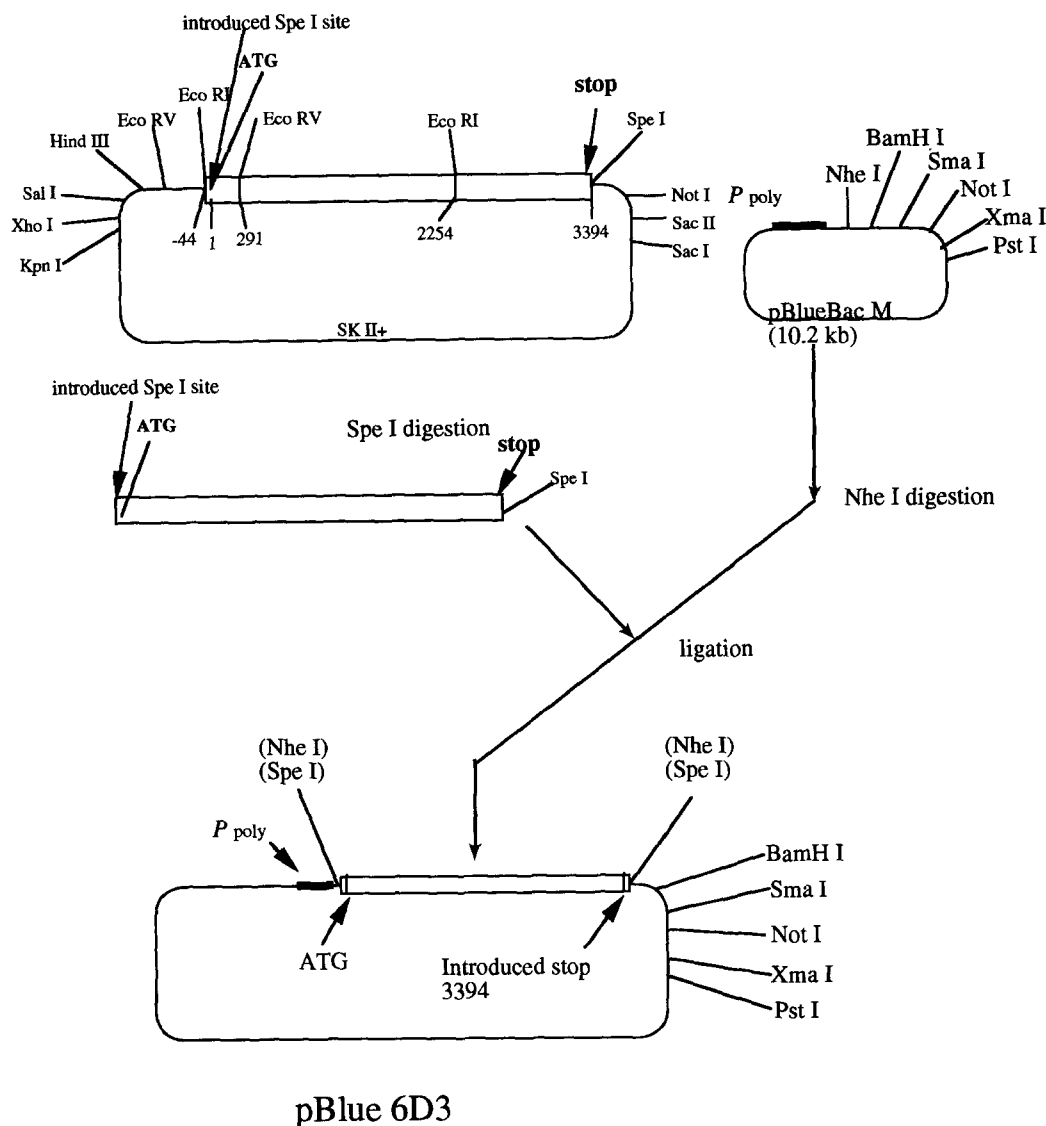

pBlue 6D3

Fig. 1. Construction of smooth muscle myosin heavy chain Baculovirus expression vector (pBlue 6D3). cDNA encoding entire open reading frame of gizzard myosin heavy chain was cut with EcoRI and SpeI and the fragment was subcloned into pBluscript SKII vector. SpeI site was introduced at −19 position and a stop codon was introduced at various sites using site directed mutagenesis strategy as described [31]. The myosin heavy chain cDNA was cut with SpeI digestion and the fragment was subcloned into pBlueBac M Baculovirus transfer vector.
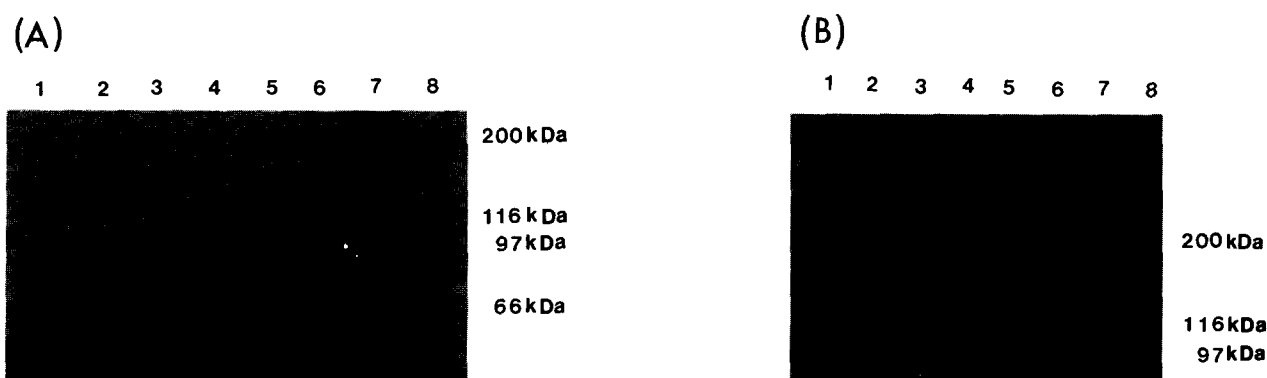
(A)

(B)

Fig. 2. SDS PAGE and immunoblots of the expressed truncated smooth muscle myosin mutants. (A) Coomassie brilliant blue stained gel. Lane 1, 97 kDa; lane 2, 100 kDa; lane 3, 108 kDa; lane 4, 110 kDa; lane 5, 114 kDa; lane 6, 128 kDa; lane 7, 134 kDa heavy chain mutants, respectively. lane 8, molecular weight standard. (B) Immunoblotting of the gel. Anti myosin heavy chain monoclonal antibody (mm-3) which recognizes the 68 kDa N-terminal domain was used as a probe. Lanes are as described above.

consistent with the expected polypeptide length, i.e. 1,153, 1,110, 991, 957, 944, 872, and 850, respectively. The authenticity of these peptide bands was confirmed by western blotting analysis (Fig. 2B) using a monoclonal antibody, mm-3, which recognizes the 68 kDa N-terminal fragment of smooth muscle S-1.

To determine whether the truncated heavy chain mutants form a two-headed structure or one-headed structure, the expressed myosin mutants were subjected to gel filtration analysis. The elution position of myosin was monitored by measuring K^+ EDTA ATPase activity of myosin (Fig. 3). The myosin containing the 134 kDa heavy chain eluted from the gel filtration column at the same position as naturally isolated gizzard HMM suggesting that the 134 kDa heavy chain forms a two-headed structure (Fig. 3). On the other hand, the 100 kDa heavy chain eluted at the elution volume similar to the naturally isolated gizzard S-1, thus forming a single-headed structure (Fig. 3). The 110 kDa heavy chain showed two active peaks. The molecular weight of these two active peaks were estimated according to the elution volumes. It was revealed that the elution volume of the first peak corresponds to the expected elution volume of a two-headed structure while that of the second peak corresponds to the elution volume of a single-headed structure. The proportion of the two-headed vs. the single-headed form of each truncated myosin heavy chain was estimated according to K^+ EDTA ATPase activity of each peak. As shown in Table 1, myosin heavy chain formed the two-

headed structure when its molecular weight was above 114 kDa while all myosin fragments below 100 kDa were the single-headed type. On the other hand, both two-headed and single-headed structures were present with the heavy chain molecular mass of 110 kDa (Fig. 3, Table 1). When the heavy chain was further truncated by 2 kDa, no obvious two-headed myosin peak was observed (Fig. 3), however, the activity peak was asymmetrical with the maximum at the position of the single-headed myosin. The result suggests that the single-headed form is dominant and that the two-headed form of the 108 kDa heavy chain is unstable and in relatively fast equilibrium with the single-headed form. To examine whether or not the double-headed structure is stable for the 110 kDa construct, the double-headed fractions were rechromatographed (Fig. 3). The activity was eluted at the position of the single-headed myosin suggesting that the association of the two heavy chains is not very stable which was different from the longer heavy chain construct which showed a stable two-headed structure (not shown). These results indicate that the residues Gln^{945}–Ser^{991} are critical for the formation of double-headed structure of myosin.

The expressed myosin heavy chain in cell homogenate was hybridized with both 20,000 Da and 17,000 Da light chains and coprecipitated with F-actin. The precipitates were dissolved with ATP containing buffer and the recombinant myosin was recovered from the supernatant after centrifugation. As shown in Table 2, the 134 kDa mutant showed significant phosphorylation-dependent actin activated ATPase activity (74 nmol/min/mg). Similar phosphorylation-dependent activity was obtained for naturally isolated HMM (data not shown, see also [15]). Myosin heavy chain (134 kDa) was also coexpressed with the 20,000 Da and 17,000 Da light chains in the insect cell line Sf9. The purified myosin fragment showed similar phosphorylation dependent actin activated ATPase activity (data not shown). While the phosphorylation-dependent activity was observed with the myosin fragments with greater than 114 kDa of heavy chain, the myosin fragments with less than 108 kDa of heavy chain showed virtually no phosphorylation dependence (Table 2). As shown in Fig. 2, the 110 kDa heavy chain formed both two-headed and one-headed myosin. The myosin fragments in each active peak of Sephacryl S-300 fractions were immediately subjected to the actin-coprecipitation step for purification (see

Table 1
Relative amount of the single-headed and double-headed forms of myosin mutants

Molecular weight of heavy chain	Relative amount of myosin (%)[a]	
	Double-headed form	Single-headed form
98 kDa	0.0	100
100 kDa	0.0	100
108 kDa	ND	ND
110 kDa	63	37
114 kDa	81	19
128 kDa	98	1.7
134 kDa	100	0.0

[a]The amount of myosin was estimated by measuring the EDTA ATPase activity of the gel filtration fractions as described in Fig. 3.
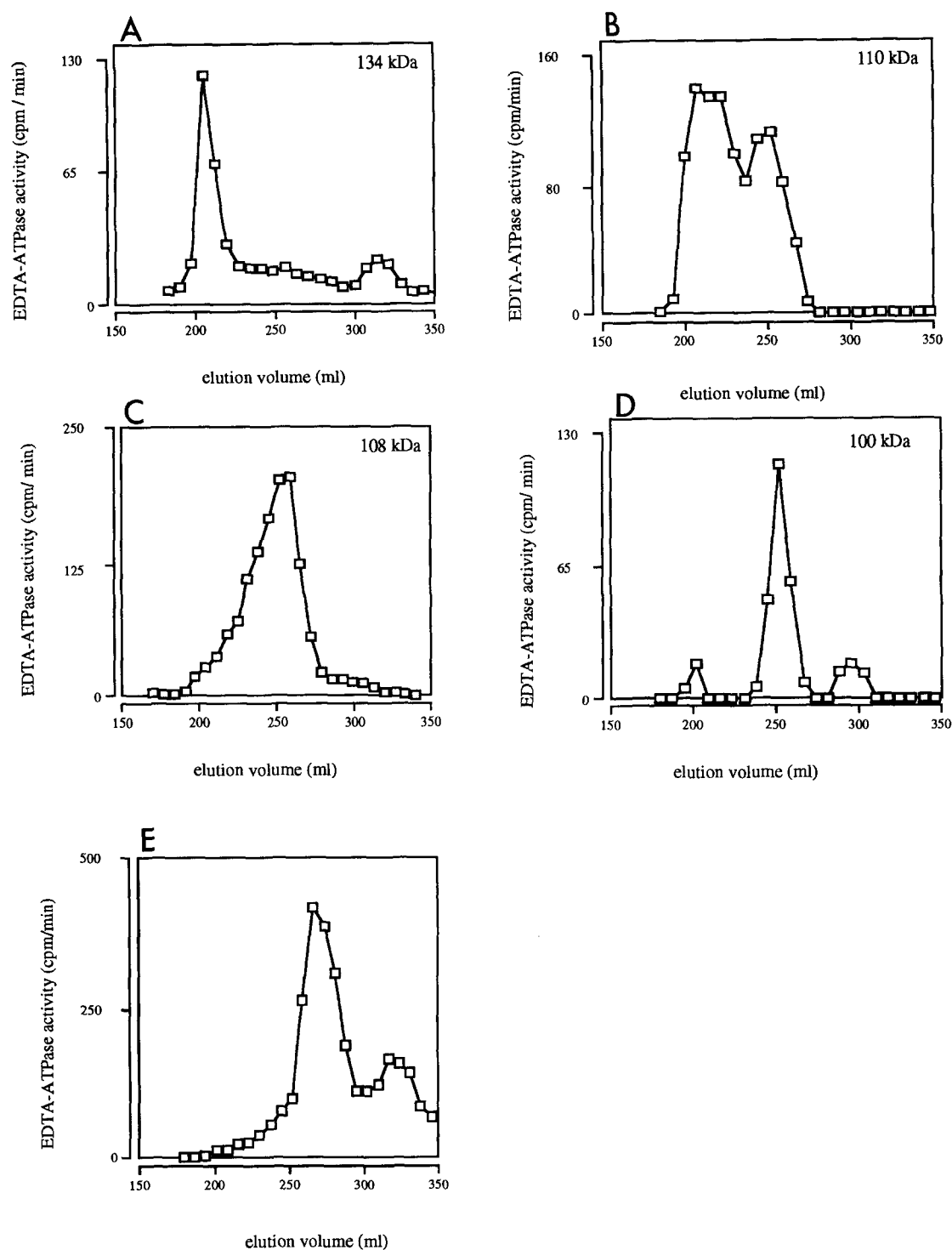
Fig. 3. Elution profile of Sephacryl S-300 HR gel filtration of various smooth muscle myosin truncation mutants. Coinfected Sf9 cells were harvested and lysed after 72 h of infection. The lysate in the presence of two classes of light chains was applied to Sephacryl S-300 HR column (2.5 cm × 100 cm) equilibrated with 0.6 M KCl, 50 mM Tris-HCl (pH 7.5), 1 mM ATP, 1 mM MgCl₂, 1 mM DTT, and 1 M monosodium glutamate. The fractions (7 ml/tube) were collected at a flow rate of 40 ml/h. EDTA-ATPase activity of each fraction was measured at 25°C in the solution containing 0.6 M KCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5 mM [γ-^{32}P]ATP (500 cpm/nmol). (A) 134 kDa mutant; (B) 110 kDa mutant; (C) 108 kDa mutant; (D) 100 kDa mutant; (E) rechromatography of the 1st peak of B.

section 2). The ATPase activity of the engineered myosins was then determined.

The single-headed fraction did not show any phosphoryla-

tion dependent ATPase activity while the double-headed fraction showed phosphorylation-dependent activity although the value was significantly lower than the longer heavy chain mu-

Table 2
Phosphorylation dependence of the actin activated ATPase activity of the truncated myosin mutants

Molecular weight of myosin heavy chain	Actin activated Mg-ATPase (nmol P_i/min, mg)[a]		
	Dephos-phorylated	Phosphorylated	Phosphorylation-dependent activity
98 kDa	90	90	0
100 kDa	92	105	13
108 kDa	99	99	0
110 kDa single-headed	95	97	2
110 kDa double-headed	81	105	23
114 kDa	63	141	78
128 kDa	51	101	50
134 kDa	44	118	74

[a]Actin activated Mg^{2+}-ATPase activity was measured at 25°C in 50 mM KCl, 30 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, with or without 3 mg/ml F-actin. 0.1 mM $Ca2^+Cl_2$, 10 μg/ml MLCK, 5 μg/ml calmodulin or 1 mM EGTA were added for phosphorylated or dephosphorylated myosin, respectively.

tants (Table 2). Considering that the longer heavy chain mutants form double-headed myosin while the shorter heavy chain mutants form single-headed myosin, one can conclude that the phosphorylation-dependent regulation requires the double-headed structure of myosin molecule. The lower extent of phosphorylation dependence of the double-headed 110 kDa heavy chain mutant is likely to be due to the low stability of the double-headed structure (Fig. 3). Quite recently while this study was underway, it was reported that [32] smooth muscle HMM with a longer tail has higher phosphorylation dependence than HMM with a shorter tail. Our data is consistent with this notion and provides further evidence for the hypothesis that the double-headed structure is critical for the phosphorylation-dependent regulation.

It is known that S-1 is active without phosphorylation, therefore, it is plausible that each myosin head itself is active without phosphorylation and the possible intra-molecular head interaction is critical for the regulation of smooth muscle myosin motor activity. When myosin is dephosphorylated the resulting head interaction may inhibit the motor activity. The results of the present study clearly support this hypothesis. It is known that the 20,000 Da light chain resides at the S-1-S-2 junction [11] and the phosphorylation of this light chain induces a conformational change at the head–rod junction [17–20]. Therefore, it may be postulated that the role of light chain phosphorylation is to change the conformation at S-1-S-2 junction so as to disrupt the interaction between the two heads which is critical for the inhibition of myosin motor activity. The understanding of the detailed mechanism of the phosphorylation mediated regulation requires further study.

References

[1] Hartshorne, D.J. (1987) in: Physiology of the Gastrointestinal Tract (Johnson, L.R. Ed.) 2nd Ed., Vol. 1, pp. 423–482, Raven Press, New York.

[2] Sellers, J.R. and Adelstein, R.S. (1987) in: The Enzymes (Boyer, P. and Krebs, E.G. Eds.) Vol. 18, pp. 381–418, Academic Press, San Diego, CA.

[3] Kamm, K.E. and Stull, J.T. (1989) Annu. Rev. Physiol. 51, 299–313.

[4] Pearson, R.B., Jakes, R., John, M., Kendrick-Jones, J., and Kemp, B.E. (1984) FEBS Lett. 168, 108–112.

[5] Ikebe, M. and Hartshorne, D.J. (1985) J. Biol. Chem. 260, 10027–10031.

[6] Ikebe, M., Hartshorne, D.J. and Elizinga, M. (1986) J. Biol. Chem. 261, 36–39.

[7] Kamisoyama, H., Araki, Y. and Ikebe, M. (1994) Biochemistry 33, 840–847.

[8] Ikebe, M., Hartshorne, D.J. and Elzinga, M. (1987) J. Biol. Chem. 262, 9569–9573.

[9] Begnur, A.R., Robinson, E.A., Appella, E. and Sellers, J.R. (1987) J. Biol. Chem. 262, 7613–7617.

[10] Nishikawa, M., Sellers, J.F., Adelstein, R.S. and Hidaka, H. (1984) J. Biol. Chem. 259, 8808–8814.

[11] Rayment, I., Rypniewski, W.R., Schmidt-Base, K., Smith, R., Tomchick, D.R., Benning, M.M., Winkelmann, D.A., Wesenberg, G., and Holden, H.M. (1993) Science 261, 50–54.

[12] Trybus, K.M. and Chatman, T.A. (1993) J. Biol. Chem. 268, 4412–4419.

[13] Trybus, K.M., Waller, G.S. and Chatman, T.A. (1994) J. Cell Biol. 124, 963–969.

[14] Ikebe, M., Reardon, S., Mitani, Y., Kamisoyama, H., Matsuura, M. and Ikebe, R. (1994) Proc. Natl. Acad. Sci. 91, 9096–9100.

[15] Ikebe, M. and Hartshorne, D.J. (1985) Biochemistry 24, 2380–2387.

[16] Ikebe, M. and Hartshorne, D.J. (1984) J. Biol. Chem. 259, 11639–11642.

[17] Morita, J., Takashi, R. and Ikebe, M. (1991) Biochemistry 30, 9539–9545.

[18] Suzuki, H., Stafford III, W.F. Slayter, H.S. and Seidel, J.C. (1985) J. Biol. Chem. 260, 14810–14817.

[19] Hartshorne, D.J. and Ikebe, M. (1987) in: Platelet Activation (Yamazaki, H. and Mastard, J.F. Eds.) pp. 3–17, Academic Press, Tokyo.

[20] Higashihara, M., Young-Frada, L.-L., Craig, R. and Ikebe, M. (1989) J. Biol. Chem. 264, 5218–5225.

[21] Ikebe, M. and Hartshorne, D.J. (1985) J. Biol. Chem. 260, 13146–13153.

[22] Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.

[23] Ikebe, M., Stepinska, M., Kemp, B.E., Means, A.R. and Hartshorne, D.J. (1987) J. Biol. Chem. 262, 13828–13834.

[24] Walsh, M.P., Hinkins, S., Dabrowska, R. and Hartshorne, D.J. (1983) Methods Enzymol. 99, 279–288.

[25] Hathaway, D.R. and Haeberle, J.R. (1983) Anal. Biochem. 135, 37–43.

[26] Ikebe, M., Inagaki, M., Naka, M. and Hidaka, H. (1988) J. Biol. Chem. 263, 10698–10704.

[27] Yanagisawa, M., Hamada, Y., Katsuragawa, Y., Imamura, M., Mikawa, T. and Masaki, T. (1987) J. Mol. Biol. 198, 143–157.

[28] Sanger, F., Nickles, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5464.

[29] O'Reilly, D.R., Miller, L.K. and Luckow, V.A. (1992) Baculovirus Expression Vectors: A Laboratory Manual, W.H. Freman and Co., New York.

[30] Laemmli, U.K. (1976) Nature 227, 680–685.

[31] Yano, K., Araki, Y., Hales, S.J., Tanaka, M. and Ikebe, M. (1993) Biochemistry 32, 12054–12061.

[32] Trybus, K.M. (1994) J. Biol. Chem. 269, 20819–20822.