CONFORMATIONAL CHANGE AND REGULATION OF MYOSIN MOLECULES

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1. DEVELOPMENT OF MYOSIN BIOCHEMISTRY

It has been a long time since myosin was first named by Wilhelm Kuhne in the mid 19th century as a component of skeletal muscle extract precipitated at low salt. In 1939, Engelhartd found that myosin is an ATPase. A critical finding was made by Albert Szent-Gyorgyi in 1942 who found that myosin interacts with actin and produces superprecipitation with the addition of ATP. The finding opened a door for the study of muscle contraction and actomyosin biology. The enzymatic function of myosin has been studied by biochemical means. One of the most important findings of myosin enzymatic function is the discovery of Pi-burst by Tonomura et al. (Tonomura et al., 1962). They found that the timecourse of the Pi release of myosin ATPase shows initial rapid phase followed by a steady state Pi release. It was thought that the initial Pi-burst reflectd the formation of phosphorylated intermediate, but it was realized later that this is due to the formation of myosin/ADP/P intermediate. The kinetic model of actomyosin ATPase subsequently developed (Lymn and Taylor, 1971; Stein et al., 1981; Siemankowaki et al., 1985), and it is now known that each kinetic step is closely correlated with the mechanical cycle of cross-bridge movement. A critical feature of myosin in addition to the ATPase activity and the actin binding activity is the filament formation. In the 1960's, a number of studies were made to determine the molecular structure of myosin using various physico-chemical techniques. The molecular shape of skeletal myosin was visualized by electron microscopy and it was found that myosin has two globular heads connected with a long tail (Slayter and Lowey, 1967) that is critical for the thick filament formation. While the filament formation is a key feature of a myosin molecule, an unusual myosin like protein was first discovered from an amoeba. Pollard and Korn (Pollard and

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Korn, 1971) isolated a myosin like protein from Acanthamoeba that has ATPase activity and the actin binding ability, but no elongated tail domain of myosin and resembles myosin S1, the head portion of muscle myosin. This unique myosin was named myosin I. During the last decade, a number of myosin like proteins were found and it is now known that myosin constitutes a superfamily (Fig. 1). The myosin superfamily is currently organized into at least 18 classes based upon phylogenetic sequence comparisons of the motor domain (Reck-Peterson et al., 2000; Mermall et al., 1998; Sellers, 2000; Berg et al., 2001). Among them,

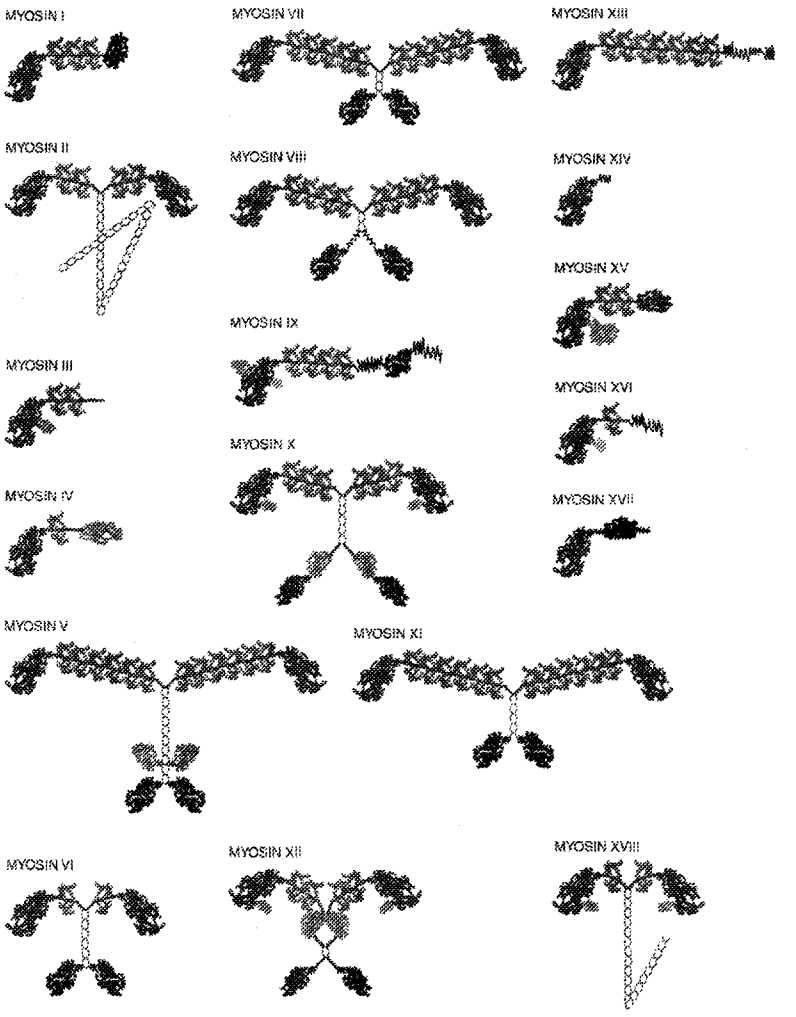

Figure 1. Schematic drawing of myosin superfamily members.

only the class II myosin (conventional myosin) can form thick filament, and it is thought that the class-specific tail domain plays a role in binding to the target molecules, thus determining the cellular localization and function of the each member of myosin.

2. REGULATION OF ACTOMYOSIN

The regulation of actomyosin was first studied with skeletal muscle. Ebashi and Ebashi (Ebashi and Ebashi, 1964) found regulatory components in skeletal actomyosin preparation and this was named troponin. A number of studies have established that troponin-tropomyosin complex, the thin filament liked regulatory components, is responsible for the Ca^{2+} dependent regulation of striated muscle actomyosin. On the other hand, a myosin-linked regulation mechanism was first observed in Molluscan muscle actomyosin (Kendrick-Jones et al., 1970). It was found that a class of light chain, called regulatory light chain (RLC), dissociates from myosin by EDTA, and this abolishes the Ca^{2+} dependent regulation of actomyosin, thus making actomyosin constitutively active. Initially, the Ca^{2+} binding site was thought to be RLC, but subsequently it was realized that the Ca^{2+} binding site is in the other light chain, essential light chain (ELC) and RLC stabilizes Ca^{2+} binding to ELC (Xie et al., 1994). The regulation of smooth muscle actomyosin is also myosin linked, but the mode of regulation is different from that of Molluascan actomyosin. It was found that smooth muscle myosin is phosphorylated in the presence, but not in the absence, of Ca^{2+}, and the phosphorylation is required for the activation of actomyosin (Sobieszek, 1977 ; Gorecka et al., 1976; Ikebe et al., 1977). Once myosin is phosphorylated, Ca^{2+} is no longer required for the activation. Furthermore, it was found that the phosphorylation changes the conformation of smooth muscle myosin. It was originally found in Watanabe's laboratory that smooth muscle myosin showed the different sedimentation velocity in high and low ionic strength. The sedimentation velocity was 6S at high salt and 10S at low salt, and it was thought that the 10S

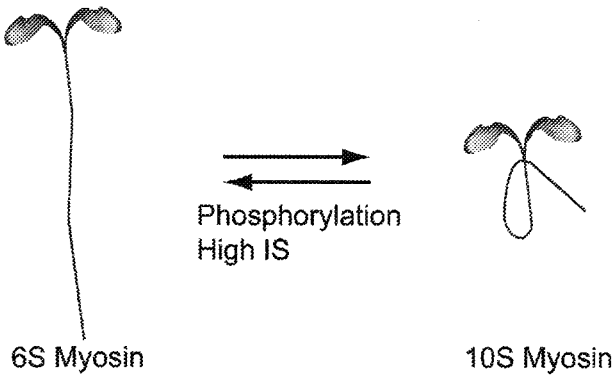

Figure 2. Schematic representation of 10 S and 6 S conformation of smooth muscle myosin II.

component is the dimer of myosin (Suzuki et al., 1978). Subsequently, it was found that this is not the case and the tail of myosin bent back towards the head-neck junction in 10S myosin (Onishi and Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983) (Fig. 2). Importantly, the phosphorylation of RLC favors the formation of 6S conformation (Onishi et al., 1983; Ikebe et al., 1983; Trybus and Lowey, 1984). Quite interestingly, we found that myosin Va undergoes similar conformational change that is accompanied by the change in the ATPase activity. The following section describes this finding in detail.

3. MYOSIN V UNDERGOES CONFORMATION TRANSITION COUPLED WITH ITS MOTOR ACTIVITY

Myosin V is an unconventional myosin that processively moves along an actin filament. Myosin V has two heads, each of which consists of a motor domain and an expanded neck domain that contains six IQ motifs that bind calmodulins or light chains. Myosin V was isolated from chicken brain and found that the actin activated ATPase activity is significantly activated by Ca^{2+} (Cheney et al., 1993). However, the recombinant truncated myosin V, having the head domain and the coiled-coil domain expressed in Sf9 cells, showed no activation of the ATPase activity in Ca^{2+} (Trybus et al., 1999; Homma et al., 2000). The reason why tissue isolated myosin Va and expressed myosin Va fragments show different properties is unknown. An obvious possibility is that the tail domain influences the Ca^{2+} dependent regulation. Alternatively, the difference may be attributed to the differences in the post translational modifications of recombinant and tissue isolated myosin Va or the subunit compositions and it is reported that tissue isolated myosin Va contains dynein light chain (Espindola et al., 2000). We succeeded in functionally expressing recombinant full-length myosin Va and studied Ca^{2+} dependent regulation of myosin Va by examining the expressed full-length myosin Va and various truncated variants.

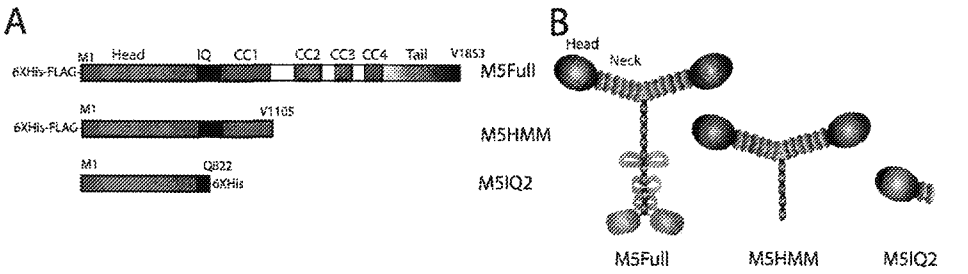

Figure 3. Schematics of Myosin Va constructs. (A) Schematic primary structure of myosin Va constructs expressed in this study. CC represents coiled-coil sequence. IQ represents the calmodulin light chain binding domain. His-Tag and FLAG-tag were added at N-terminus of M5Full and M5HMM, while His-Tag was added at C-terminus of M5IQ2. (B) Schematic structure of myosin Va constructs based on its amino acid sequence. The chain represents coiled-coil structure.

3.1. Expression of recombinant full-length myosin Va and its fragments.

We succeeded in functionally expressing a full-length mouse myosin Va (M5Full). Myosin Va heavy chain was co-expressed with calmodulin light chain and purified as described in MATERIALS AND METHODS (Fig. 3). The expressed M5Full was composed of heavy chain with apparent molecular weight of 190 kDa and calmodulin light chain. Myosin Va HMM (M5HMM) containing the entire head, IQ domain and coiled-coil domain, and M5IQ2 containing entire head and 2 IQ domains were also isolated. These constructs were used for the experiments described in this study.

3.2. ATPase activity of recombinant Myosin Va and its fragments.

Fig. 4A shows the actin-activated ATPase activity of M5Full in the presence and absence of free Ca^{2+}. The actin-activated ATPase activity of M5Full showed strong Ca^{2+} dependence and the activity was increased for approximately 9 folds in the presence of 100 μM free Ca^{2+} (Fig. 4A and Table I). The result of M5Full is similar to that of tissue-isolated myosin Va (Cheney et al., 1993). On the other hand, Ca^{2+} did not significantly change the activity of M5HMM in the presence of 12 μM calmodulin (Fig. 4B). In the absence of exogenous calmodulin, actin-activated ATPase activity of M5HMM was decreased at high Ca^{2+} as was reported previously (Homma et al., 2000), and it is thought that the decrease in the ATPase activity is due to the dissociation of calmodulin light chain. The ATPase activity of M5IQ2 showed reverse Ca^{2+} dependence and the activity was slightly higher in EGTA than in Ca^{2+} (Fig. 4C). Both truncated constructs have high ATPase activity in EGTA condition, which is similar to that of M5Full in Ca^{2+} condition. The results suggest that the Ca^{2+} dependent

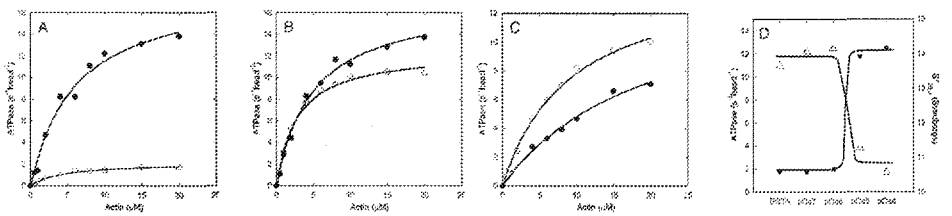

Figure 4. MgATPase activity of myosin Va constructs. (A-C). The actin dependence of ATPase activity of M5Full (A), M5HMM (B), and M5IQ2 (C) in EGTA (open circle) and pCa4 condition (closed circle). The solid lines in (A-C) are fit to the Michaelis-Menten equation. The fitting data are summarized in Table I. (D) Effects of Ca^{2+} on the ATPase activity (closed circle) and sedimentation coefficient (open triangular) of M5Full. The ATPase activity was measured at 25°C in a solution of 20 mM MOPS (pH7.0), 0.1 M KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.25 mg/ml BSA, 12 μM calmodulin, 20 μM actin, 0.5 mM ATP, 2.5 mM PEP, 20 U/ml pyruvate kinase. EGTA (1 mM) was added for EGTA condition, whereas 1 mM $CaCl_2$ and various concentrations of EGTA were added for pCa7-pCa4 conditions.

Table 1. V_{max} and K_{actin} of Actin-activated ATPase activity of Myosin Va

	EGTA condition			Ca^{2+} condition		
	V_0 ($s^{-1}head^{-1}$)	V_{max} ($s^{-1}head^{-1}$)	K_{actin} (μM)	V_0 ($s^{-1}head^{-1}$)	V_{max} ($s^{-1}head^{-1}$)	K_{actin} (μM)
M5Full	0.05	2.15	4.38	0.06	18.52	6.02
M5HMM	0.09	12.48	2.75	0.09	17.30	4.84
M5IQ2	0.07	15.07	9.21	0.07	14.45	19.87

Assay conditions were as described in the legend to Figure 1. Basal activity (V_0) was deducted. Curves are the least squares fits of the data points based upon the equation: $V = (V_{max} * [actin]) / (K_{actin} + [actin])$.

regulation observed for M5Full is due to the inhibition in EGTA rather than the activation in Ca^{2+}. Figure 4D shows free Ca^{2+} concentration dependence of the actin activated ATPase activity of M5Full. The activity was increased at higher than pCa5 suggesting that Ca^{2+} binding to calmodulin light chain is responsible for the activation. The result suggests that the tail domain of myosin Va is responsible for Ca^{2+} dependent regulation.

3.3. Ca^{2+} dependent conformational change of Myosin Va.

We examined the conformational changes of myosin Va constructs. First, we analyzed the sedimentation coefficient ($S_{20,w}$) of myosin Va in EGTA and Ca^{2+} conditions by velocity sedimentation analysis. The apparent $S_{20,w}$ of myosin Va decreased significantly from 13.9 S in EGTA to 11.3 S in pCa5 condition (Fig 5; Table 2). Remarkably, the stimulation of actin-activated ATPase activity of M5Full by Ca^{2+} is accompanied with the decrease of $S_{20,w}$ (Figure 4D; Table 2).

M5HMM, containing partial coiled-coil domain, failed to show a decrease of $S_{20,w}$. Therefore, the results suggest that the observed change in the $S_{20,w}$ of M5Full is due to the large change in the conformation. It is predicted that M5Full forms a more compact conformation in the presence of low Ca^{2+} than in high Ca^{2+}. On the other hand, the $S_{20,w}$ of M5Full at high ionic strength was slightly increased from 9.4 in EGTA to 9.7 in pCa5 (Table 2). Both values were similar to that in Ca^{2+} at low ionic strength, suggesting that the formation of a compact structure is abolished at high ionic strength. In contrast to M5Full, the $S_{20,w}$ for M5HMM rather increased from 8.7 S in EGTA to 9.5 S in Ca^{2+} condition (Fig. 5; Table 2). Ca^{2+} induced the slight decrease of $S_{20,w}$ with M5IQ2 (Fig. 5; Table 2). While the change was small, the same results were obtained repeatedly, therefore, the change in $S_{20,w}$ of M5IQ2 may reflect the Ca^{2+} induced release of the bound calmodulin (Homma et al., 2000).

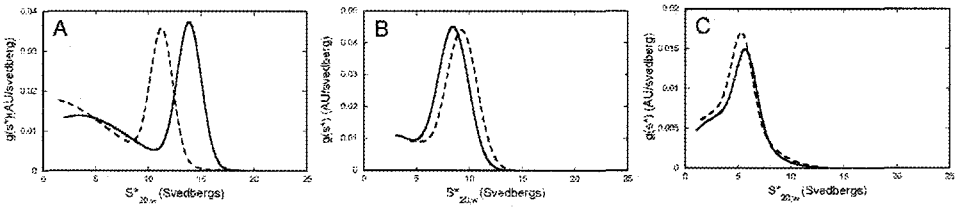

Figure 5. Apparent sedimentation coefficient distributions for myosin Va constructs. Sedimentation velocity was determined in solutions containing 20 mM MOPS (pH7.0), 0.2 M NaCl, 1 mM DTT, 1 mM EGTA (solid line). EGTA was replaced by EGTA-$CaCl_2$ buffer for pCa5 condition (broken line). The velocity runs were carried out at 42,000 rpm at 20°C for M5Full (A), M5HMM (B), and M5IQ2 (C). The x axis, $S^*_{20,w}$ is the apparent sedimentation coefficient.

Table 2. Sedimentation coefficient ($S_{20,w}$)of myosin Va constructs.

	EGTA	pCa7	pCa6	pCa5	pCa4
M5Full*	13.9	14.1	14.2	11.3	10.6
M5Full**	9.4	\	\	9.7	\
M5HMM*	8.7	\	\	9.5	\
M5IQ2*	5.8	\	\	5.4	\

* Analytical centrifugation was run in a solution of 20 mM MOPS (pH7.0), 0.2 M NaCl, 1 mM $MgCl_2$, 1 mM DTT and various concentration of EGTA and $CaCl_2$ as described in Materials and Methods. ** Same condition as * except 0.6 M NaCl was used.

To visualize the nature of the conformational change of M5Full, we examined the structure of M5Full by rotary shadowing of electron microscopy. Fig. 6 shows the representative images of M5Full in various conditions. At high ionic strength at low Ca^{2+}, M5Full showed an extended conformation that was similar to those images previously reported (Cheney et al., 1993). On the other hand, we found a folded shape of M5Full at low ionic strength in the presence of EGTA, in which the tail domain was folded back towards the head-neck region. In the Ca^{2+} condition, we predominantly found an extended conformation even at low ionic strength. These results are consistent with centrifugation analysis and show that 14 S M5Full represents a folded conformation, while 11 S M5Full represents an extended conformation. Furthermore, we found that the head of myosin Va appears large and globular with no obvious neck domain in high Ca^{2+} regardless of ionic strength. On the other hand, in low Ca^{2+} myosin Va showed a smaller globular head connected with long neck domain (Fig 6).

We found that myosin Va (M5Full) exhibits a Ca^{2+} dependent large change in the $S_{20,w}$ at physiological ionic strength. Based upon rotary shadowing of electron microscopy, it was found that M5Full forms a folded conformation at low Ca^{2+} and low ionic strength. In this conformation, myosin Va tail bent back to the head-neck region of the molecule. On the other hand, M5Full forms a more extended conformation at high Ca^{2+}. At high ionic strength, an extended conformation dominates over a folded conformation regardless of the Ca^{2+} concentration that is reflected by the decrease in $S_{20,w}$. Ca^{2+} concentration required for the

shift in conformation is pCa6-pCa5 based upon the change in the $S_{20,w}$. This suggests that the conformational change is initiated by the binding of Ca^{2+} to calmodulin light chain that is associated at the neck of myosin Va. It should be mentioned that myosin Va showed several apparently different shapes in low Ca^{2+} at low ionic strength, although the molecules showed compact structures. This suggests that the attachment of the tail domain to the neck domain is not tight and there is enough flexibility to allow myosin Va molecules to take various conformations.

We also found that Ca^{2+} changes the head-neck conformation regardless of the ionic strength. At high Ca^{2+}, the characteristic long neck domain of myosin Va is not evident and two large globular heads are observed. We think that the globular motor domain of myosin Va is folded back to the neck domain at high Ca^{2+} to create apparent large globular head (Fig 6). At low ionic strength, the attachment of the motor domain to the neck domain in Ca^{2+} may prevent the interaction of the tail domain at the neck domain thus inhibiting the formation of a folded conformation. The Ca^{2+} induced increase in $S_{20,w}$ with M5Full in high salt (9.4S to 9.7S) and M5HMM (8.7S to 9.5 S) supports the model (Fig. 6B) in which the head folds back to neck in high Ca^{2+}.

Interestingly, the change in the conformation of M5Full was closely correlated with the change in the actin-activated ATPase activity. At low ionic strength, Ca^{2+} markedly increases the actin-activated ATPase activity and this is accompanied by the change in the conformation from a folded to an extended. On the other hand, there was no change in the ATPase activity by Ca^{2+} at high ionic strength, where the compact folded structure of myosin Va is not found. A similar relationship between the conformational change and ATPase activity of myosin has been known for vertebrate smooth muscle and non-muscle myosin II as described above (Onishi et al., 1983; Ikebe et al., 1983; Trybus et al., 1984).

A folded myosin II has a low ATPase activity while an extended myosin II shows significantly higher ATPase activity (Ikebe et al., 1983). The change in the conformation of myosin II is regulated by regulatory light chain phosphorylation (Onishi et al., 1983; Ikebe et al., 1983; Trybus et al., 1984) and it is thought that the phosphorylation of the light chain induces the conformational change at the neck region where the light chain associates and this stabilizes the association of the tail domain to form a folded conformation. For myosin Va, it is anticipated that Ca^{2+} binding to calmodulin light chain induces the conformational change of calmodulin at the neck domain, which destabilizes the association of the tail domain to the head-neck region of myosin Va.

Recently, a similar tail inhibition model was proposed for the regulation of kinesin, i.e., kinesin is in a folded conformation such that the kinesin globular tail domain interacts with and inhibits the kinesin motor activity (Verhey and Rapoport, 2001). Full-length kinesin undergoes a 9S to 6S conformational transition, i.e. compact to extended conformation, whereas C-terminal domain truncated kinesin constitutively in extended form (Stock et al., 1999). Correspondingly, the ATPase activity of full-length kinesin is activated by cargo binding, whereas C-terminal domain truncated kinesin is constitutively active. Furthermore, it was found that the expressed C-terminal globular domain inhibits the ATPase activity of C-terminal domain truncated kinesin (Coy et al., 1999). The present study suggests that there is a similarity in the regulatory mechanism between kinesin and myosin Va.

A question is whether the myosin V motor function is regulated via a conformational transition found in this study in cells. A critical issue is whether the myosin driven cargo movement is regulated by the change in the cytosolic Ca^{2+}, and it requires further study to clarify this question. Another issue is whether the myosin V targeting molecules affect the motor activity of myosin V since the myosin V binding partners are bound to the C-terminal globular domain of myosin V. It is plausible that the binding of the tail domain to partner molecules may release the inhibition of the ATPase, thus regulating myosin Va motor function. It requires further study to see whether cargo binding at the tail domain can activate myosin Va motor function.

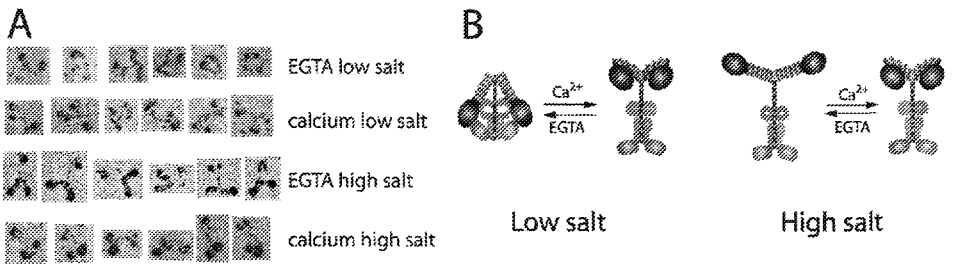

Figure 6. Electron micrographs M5Full and model for the regulation of M5Full.
A. Electron microscopic images of M5Full in various conditions. M5Full samples diluted to about 4 nM by dilution buffer were absorbed onto a freshly cleaved mica surface for 30 s. Unbound proteins were rinsed away, and then the specimen was stabilized by brief exposure to uranyl acetate. Dilution buffers were as follows: EGTA-low salt, 1 mM $MgCl_2$, 30% glycerol, 1 mM EGTA, 50 mM ammonia acetate (pH7.0); Ca^{2+}-low salt, 1 mM $MgCl_2$, 30% glycerol, 0.1 mM $CaCl_2$, 50 mM ammonia acetate (pH7.0); EGTA-high salt, 1 mM $MgCl_2$, 30% glycerol, 1 mM EGTA, 600 mM ammonia acetate (pH7.0); Ca^{2+}-high salt, 1 mM $MgCl_2$, 30% glycerol, 0.1 mM $CaCl_2$, 600 mM ammonia acetate (pH7.0). B. Schematic model of M5Full conformations. The motor domain is folded back to associate the neck domain in high Ca^{2+}. At low salt condition, the tail globular domain interacts with the head-neck region to produce a compact structure in low Ca^{2+}. This is inhibited in high Ca^{2+} because the binding of the motor domain to the neck domain interferes the association between the tail and the head-neck of myosin Va.

4. MATERIALS AND METHODS

4.1. Construction of Myosin Va expression vector.

A baculovirus transfer vector for mouse myosin Va (M5Full) in pFastBac (Invitrogen, CA) was produced as follows. A unique SpeI site was created at nucleotide 3316 of DHM5 (Homma et al., 2000). A cDNA fragment 3316-5602 franked by SpeI and KpnI sites were introduced to DHM5 to produce a full-length myosin Va construct. The nucleotides at the created SpeI site were changed to resume the authentic sequence. An N-terminal tag (MSYYH HHHHH DYKDD DDKNI PTTEN LYFQG AMGIR NSKAY) containing a sequence of hexa-histidine-Tag and FLAG-tag was added to the N-terminus of M5Full. For M5HMM, a stop codon was introduced at the nucleotide 3316. Vector containing nucleotide 1-2468 of myosin Va (M5IQ2) was prepared as described previously (Homma et al., 2000).

4.2. Expression and purification of Myosin Va.

To express recombinant M5Full, Sf9 cells (about $1X10^9$) were co-infected with two separate viruses expressing the myosin Va heavy chain and calmodulin, respectively, and the recombinant myosin Va was purified as described (Homma et al., 2000). All concentrations of Myosin Va in this paper are referred to the concentration of head.
ATPase assay: Since myosin Va ATPase activity is significantly inhibited by the product ADP, we use an ATP regeneration system to measure its activity. The MgATPase activity was measured at 25°C in a solution containing 10 - 100 nM Myosin Va, 20 mM MOPS (pH7.0), 1 mM $MgCl_2$, 0.25 mg/ml BSA, 1 mM DTT, 2.5 mM PEP, 20 U/ml pyruvate kinase, 12 μM calmodulin, 100 mM KCl, 0.5 mM ATP, 1 mM EGTA-$CaCl_2$ buffer system, and various concentrations of actin.

4.3. Analytical Ultracentrifugation.

The myosin Va sample was dialyzed against a buffer of 20 mM MOPS (pH7.0), 0.2 M NaCl, 0.1 mM EGTA, 1 mM DTT. Just before running, $MgCl_2$ and EGTA concentration was adjusted to 1 mM for EGTA condition. For Ca^{2+} condition, $MgCl_2$ and $CaCl_2$ were adjusted to 1 mM with various EGTA concentrations.
Sedimentation velocity was run at 42,000 rpm at 20°C for 2 hours in a Beckman Optima XL-I analytical ultracentrifuge. Sedimentation boundaries were analyzed using a time-derived sedimentation velocity program, i.e. DcDt (Stafford, 1992) or DcDt plus (Philo, 2000). The density and viscosity calculated with Sednterp for a solution containing 0.2 M NaCl and 1 mM $MgCl_2$ were 1.00658 g/ml and 1.0213 cp, respectively. Those values for a solution of 0.6 M NaCl and 1 mM $MgCl_2$ were 1.0228 g/ml and 1.0590 cp, respectively. The values of the partial specific volumes of M5Full (0.7323), M5HMM (0.7296), and M5IQ2 (0.7328) were calculated from the amino acid composition of heavy chain and calmodulin.

4.4. Electron microscopy.

M5Full samples diluted to about 4 nM were absorbed onto a freshly cleaved mica surface. Unbound proteins were rinsed away, and then the specimen was stabilized by brief exposure to uranyl acetate. The specimen was visualized by the rotary shadowing technique according to Mabuchi (Mabuchi, 1991) with an electron microscope (Philips 300 electron microscope) at 60 kV.

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DISCUSSION

Pfittzer: How do you think about the stabilization of smooth muscle myosin filament?

Ikebe: Dephosphorylation induces smooth muscle myosin filament formation in vitro. Whether or not myosin filament formation in intact smooth muscle is regulated is unclear, but it is believed that majority of myosin forms filament in vivo, even in a resting state. Spacio-temporal change in dephosphorylated myosin in smooth muscle after stimulation is under investigation.