Phosphorylation of Smooth Muscle Myosin Heads Regulates the Head-induced Movement of Tropomyosin*

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It has been shown that skeletal and smooth muscle myosin heads binding to actin results in the movement of smooth muscle tropomyosin, as revealed by a change in fluorescence resonance energy transfer between a fluorescence donor on tropomyosin and an acceptor on actin (Graceffa, P. (1999) Biochemistry 38, 11984–11992). In this work, tropomyosin movement was similarly monitored as a function of unphosphorylated and phosphorylated smooth muscle myosin double-headed fragment smHMM. In the absence of nucleotide and at low myosin head/actin ratios, only phosphorylated heads induced a change in energy transfer. In the presence of ADP, the effect of head phosphorylation was even more dramatic, in that at all levels of myosin head/actin, phosphorylation was necessary to affect energy transfer. It is proposed that the regulation of tropomyosin position on actin by phosphorylation of myosin heads plays a key role in the regulation of smooth muscle contraction. In contrast, actin-bound caldesmon was not moved by myosin heads at low head/actin ratios, as uncovered by fluorescence resonance energy transfer and disulfide cross-linking between caldesmon and actin. At higher head concentration caldesmon was dissociated from actin, consistent with the multiple binding model for the binding of caldesmon and myosin heads to actin (Chen, Y., and Chalovich, J. M. (1992) Biophys. J. 63, 1063–1070).

Muscle contraction is due to the parallel sliding of actin thin filaments and myosin thick filaments during the actin-activated myosin ATPase activity, their movement is powered by the hydrolysis of ATP, which provides energy for the contraction. The sliding movement is due to the cyclic interaction of myosin heads from the thick filament with actin in the thin filament and the contraction is switched on by Ca$^{2+}$ infusion into the muscle cell. In skeletal muscle, Ca$^{2+}$ binding to the troponin complex, which is bound to tropomyosin and actin in the thin filament, relieves the inhibition of the actomyosin ATPase activity. It is thought that the inhibition is due to some type of steric blocking of the myosin binding site on actin by tropomyosin. Tropomyosin is a coiled-coil dimer that binds end-to-end on the actin filament, each molecule spanning seven actin subunits. It is thought that the movement of tropomyosin away from this blocking position, initiated by the binding of Ca$^{2+}$ to troponin, removes the inhibition of contraction (reviewed in Ref. 1). Interaction of each tropomyosin molecule with seven actin subunits and end-to-end tropomyosin interaction on the thin filament imparts cooperativity to the switching process. The initial binding of myosin heads to the thin filament is also thought to take part in the switching on of skeletal muscle contraction by a shifting of the dynamic equilibrium of tropomyosin between on and off states toward the on state (reviewed in Refs. 2 and 3). Such a process could also involve a movement of tropomyosin. Recent electron microscopic studies of skeletal muscle actin thin filaments have given strong evidence to support these ideas (4–7). These studies have shown that for the full movement of skeletal tropomyosin, i.e. for the myosin binding site on actin to be fully exposed, it is necessary that Ca$^{2+}$ binds to troponin and that some myosin heads bind to actin.

Smooth muscle contraction is initiated by the Ca$^{2+}$-dependent phosphorylation of light chains bound to the head region of myosin in the thick filament and switched off by dephosphorylation (reviewed in Ref. 8). The basis of this control is that the actin-activated myosin ATPase activity is dramatically elevated by myosin phosphorylation. The molecular mechanism of this phosphorylation-dependent activation is not understood. It is also unknown what role the thin filament plays in regulation, since Ca$^{2+}$ regulation takes place in the thick filament and the smooth muscle actin thin filament does not contain tropinin. The thin filament contains a smooth muscle isoform of tropomyosin and two smooth muscle-specific proteins, caldesmon and calponin. It is known that one function of smooth muscle tropomyosin is to elevate (or potentiate) the actin-activated myosin ATPase activity (reviewed in Refs. 9–12). However, the mechanism of this potentiation is unknown. Although caldesmon and calponin control the in vivo smTm$^+$-actin-activated myosin ATPase activity, their in vivo function is uncertain (13).

Although myosin phosphorylation is necessary to switch on smooth muscle contraction, the resulting force produced and the level of phosphorylation are not uniquely coupled (reviewed in Refs. 14–22). Thus, it has generally been considered that there are additional mechanism(s) of regulation or modulation.

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‡ The abbreviations used are: smTm, gizzard smooth muscle tropomyosin; FRET, fluorescence resonance energy transfer; skS1 or skHMM, single- or double-headed, respectively, fragment of skeletal muscle myosin; smS1 or smHMM, single- or double-headed, respectively, fragment of gizzard smooth muscle myosin; smHMM-SP, unphosphorylated smHMM; smHMM-P, thiol phosphorylated smHMM; Mops, 3-(N-morpholino)propanesulfonic acid; IAEDANS, 5-[(2-iodoacetyl)amino]ethyl)amino)naphthalene-1-sulfonic acid; AEDANS, 5-((2iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; AED, IAEDANS after reaction with a sulfhydryl group; DAB, N4-di(4-dimethylamino)phenylazo)-phenyliodine; AED-DAB, AED-DAB heterodimer labeled at Cys$^{95}$ of the β-chain with IAEDANS; DAB-A, actin labeled at Cys$^{74}$ with DAB; TT, dithiothreitol; AED-DTT, IAEDANS reacted with a high excess of DTT; CaD, caldesmon; AED-CaD, CaD labeled at Cys$^{800}$ with IAEDANS; ATPY, adenosine 5′-O-(3-thiotriphosphate).
of contraction, mechanisms that might reside in the thin filament. Since it is fairly well established that the movement of skeletal muscle tropomyosin plays a key role in the thin filament regulation of skeletal muscle contraction, the question arises as to whether such a movement of smooth muscle tropomyosin also exists. In our previous work, using fluorescence resonance energy transfer (FRET), we found that, indeed, smooth muscle tropomyosin moved upon the binding to actin of single-headed (skS1) and double-headed (skHMM) fragments of skeletal muscle myosin and the single-headed (smS1) fragment of smooth muscle myosin (23). Since smS1 is constitutively active (reviewed in Ref. 24) (i.e. its ATPase activity is activated by smTm-actin whether phosphorylated or not), in the present study we examined the movement of smooth muscle tropomyosin by the double-headed (smooth muscle heavy meromyosin; smHMM) fragment of smooth muscle myosin whose activity is regulated by phosphorylation. We found that the smHMM-induced movement of tropomyosin was controlled by the phosphorylation of smHMM, suggesting the involvement of the movement of tropomyosin in the switching on and off of smooth muscle contraction. Indeed, an early x-ray diffraction study suggested that the activation of smooth muscle led to the movement of tropomyosin (25).

EXPERIMENTAL PROCEDURES

Unless stated otherwise, the procedures used in this study are those described in the previous work (23). In those cases where the Lowry assay was used for protein determination in the previous work, I have substituted the BCA assay (27) in this study. The BCA protein assay reagent A was purchased from Pierce. Previously, I have prepared G-actin for labeling with the acceptor DAB by homogenizing an actin pellet, prepared from acetone powder according to Spudich and Watt (28), with G buffer plus a 1 mM concentration of the reducing agent DTT and then dialyzing extensively versus G-buffer to remove the DTT. Labeling of this preparation always resulted in a DAB/actin ratio of about 0.7, significantly less than the maximum of 1 (23). In this work, the actin pellet was homogenized with G buffer plus 10 mM DTT and incubated 30–60 min before dialysis, which ultimately resulted in a labeling ratio very close to the maximum of 1. Smooth muscle myosin from chicken gizzard was thiophosphorylated according to Trybus and Lowry (32) with ATP-S from Sigma. The degree of thiophosphorylation was assayed by urea gel electrophoresis (30, 31) and found to be between 70 and 100% as judged by scanning and densitometry of the urea gel. The thiophosphorylated double-headed fragment of smooth muscle myosin, smHMM, was prepared by chymotryptic digestion of thiophosphorylated myosin according to Suzuki et al. (32) and will be referred to as smHMM-SP. Thiophosphorylation was used to avoid dephosphorylation by myosin phosphatase, which sometimes contaminates smooth muscle myosin preparations. The smHMM molecular mass was taken as 334 kDa, and its concentration was obtained from the absorbance at 280 nm minus that at 320 nm using an extinction coefficient \( A_{280}^{\text{nm}} = 6.4 \, \text{cm}^{-1} \).

Caldesmon (CaD) from chicken gizzard (33) and pig stomach (34) was prepared and labeled at Cys\(^{366}\) with IAEDANS (35, 36) as described previously. Cys\(^{319}\) of chicken gizzard CaD was disulfide cross-linked to Cys\(^{774}\) of actin with dithiobis(2-nitrobenzoic acid) as we have reported (33).

FRET was determined by the change in steady-state fluorescence of the AED donor attached to Cys\(^{366}\) of smooth muscle tropomyosin (AED36smTm) in the presence of the DAB acceptor attached to actin Cys\(^{774}\) (DAB-A) as a function of smHMM, as described (23). Measurements were made at 20 °C in 40 mM NaCl, 5 mM MgCl\(_2\), 5 mM Mops, 5 mM DTT, pH 7.5, with AED36smTm at 0.6 \( \mu \text{M} \) and DAB-A at 4.8 \( \mu \text{M} \). Fluorescence changes were first corrected for changes that were not due to energy transfer. These corrections were all very small or negligible as in the previous work. First of all, the direct effect of actin on the fluorescence of AED36smTm was insignificant. Second, the drop in AED36smTm fluorescence due to the trivial absorption (57) of excitation and emission light by the DAB-A was small at about 5%. This was determined in two different ways, which gave the same value. It was either calculated (37) from the change in optical density at the 340-nm excitation and 505-nm emission wavelengths upon the addition of DAB-A to unlabeled smTm or determined from the change in fluorescence of AED-DTT in the presence of smTm upon the addition of DAB-A. Finally, fluorescence was corrected for the direct effect of smHMM on the fluorescence of AED36smTm or due to trivial absorption by smHMM either by direct absorption or by increased light scattering due to its interaction with smTm-actin. These corrections were determined in two different ways and in both cases found to be insignificant. In the first, the fluorescence of AED36smTm in the presence of unlabeled actin was titrated with smHMM. In this case, fluorescence changes were the result of both a direct effect of smHMM on AED36smTm fluorescence and trivial absorption due to smHMM. In the second case, the fluorescence of AED-DTT in the presence of unlabeled smTm and actin was titrated with smHMM. In this case, changes in fluorescence were due only to changes in trivial absorption. In both cases, after small corrections for dilution due to small volumes of added smHMM, there was no significant change in fluorescence. Therefore, smHMM (thiophosphorylated or not) had no significant direct effect on the fluorescence of AED36smTm or indirect in the absence of actin due to trivial absorption. It was also necessary to check the effect of smHMM on the absorption spectrum of DAB bound to actin in the presence of unlabeled Tm. Any change in the absorption spectrum would change the overlap integral and thus change FRET (see Ref. 23 and references therein). We found that both smHMM-SP and skS1 had no effect on the absorption spectrum of DAB-actin, thus ruling out such a change as responsible for the changes in FRET that we observe. Since the energy transfer depends on the sixth power of the overlap integral, only a large change in DAB absorption would have a significant effect.

RESULTS

As in the previous study (23) the movement of smooth muscle tropomyosin by myosin heads was monitored by measuring the change in distance between a fluorescent AED donor attached to tropomyosin and a DAB acceptor (which is nonfluorescent) attached to Cys\(^{774}\) of actin (DAB-A), using the technique of fluorescence resonance energy transfer. We used chicken gizzard smooth muscle tropomyosin which is a coiled-coil heterodimer of an \( \alpha \)-chain with a single cysteine at position 190, in the C-terminal half of the molecule, and a \( \beta \)-chain with a single cysteine at position 36, near the N terminus, both chains containing 284 residues. In this work, the donor was attached to Cys\(^{366}\) of the heterodimer, since in the previous work (23) the donor at this residue showed a larger change in fluorescence than that at Cys\(^{190}\).

Upon the addition of DAB-A to AED36smTm, there was a drop in AED fluorescence due to a transfer of energy to DAB on actin (Fig. 1). This transfer corresponded to a distance of about 45 Å between the donor and acceptor in the smTm-actin complex, assuming energy transfer between a single donor and single acceptor, close to the 43 Å we reported earlier (23). The further addition of thiophosphorylated smHMM (smHMM-SP), in the absence of nucleotide, resulted in an additional drop in AED fluorescence (Fig. 1), corresponding to a decrease in distance between tropomyosin donor and actin acceptor of about 4.5 Å and thus to a movement of tropomyosin. This is roughly the same change in distance brought about by the single-headed smooth muscle myosin fragment smS1 (23). The head-
induced change in fluorescence was measured as a function of added thiophosphorylated and unphosphorylated smHMM (smHMM-UP). A comparison of these titrations demonstrated that smHMM-UP did not change fluorescence up to a smHMM-UP head/actin ratio of 1:7, whereupon the fluorescence dropped with a further increase in smHMM-UP concentration, whereas smHMM-SP showed no such lag (Fig. 2). Both smHMM-SP and smHMM-UP bound to the AEDsmTm-DAB-A complex, determined under the same exact conditions used for fluorescence measurements, close to 100% at all levels of head/actin used in the titration. Thus, the lag in FRET change with smHMM-UP is not due to a lag in its binding to actin.

In the presence of ADP, the difference between smHMM-SP and smHMM-UP was even more dramatic (Fig. 3). While smHMM-SP showed roughly the same change in FRET as in the absence of ADP, smHMM-UP essentially had no effect on FRET at all levels of head/actin tested. Although ADP had no effect on the binding of smHMM-SP to donor-smTm/acceptor-actin, ADP did weaken the binding of smHMM-UP (Table I). The percentage binding of smHMM-UP increased as the smHMM head/actin ratio increased (Table I), indicating a positive cooperativity. However, this weakened binding does not explain the lack of effect of smHMM-UP on FRET, since, for example, at a smHMM-UP added head/actin ratio of 6:7, greater than 90% of smHMM is bound to actin, and yet there is essentially no change in FRET (Fig. 3). The fluorescence dependence on smHMM-SP concentration shows a leveling off at a head/actin ratio of about 4:7 (Fig. 2), lower than saturation at 7:7 but considerably higher than the 1:7 for smS1 (23). The difference in the steepness of the change in fluorescence between smHMM-SP and smS1 is similar to the difference between the skeletal muscle myosin single- and double-headed fragments (23), indicating that this difference is due to single- and double-headed moieties. Thus, it appears that the double-headed myosin fragments affect the tropomyosin less cooperatively than the single-headed fragments.

To quantify this difference in cooperativity, we employed the same model (38) that we used in the analysis of the movement of smTm by skS1 in our previous study (23). In this model, it is assumed that: 1) there is random binding of HMM or S1 to actin monomers in the thin filament; 2) HMM binds to two actin subunits; and 3) the binding of more than one S1 or HMM within a cooperative unit has no further effect on the movement of tropomyosin. There is evidence that for strong-binding heads (i.e. at low salt and in the absence of nucleotide), both heads of HMM bind to actin subunits (see Ref. 39 and references therein), and S1 or HMM molecules bind randomly (i.e. as a linear function of head concentration) to actin (38, 40, 41). We used the equation $f_m = 1 - (1 - f_b)^n$ (38), where $f_m$ is the fraction of tropomyosin molecules moved, $f_b$ is the fraction of actin monomers to which S1 or HMM is bound, and $n$, the cooperative unit, is the number of actin monomers affected, via the movement of tropomyosin, by the binding of each S1 or HMM molecule to the actin thin filament. Several curves were generated for this equation, using multiples of 7 for $n$, and compared with data from Fig. 2 of this work or Fig. 4 of Ref. 23, assuming that the fractional change in AED36smTm fluorescence corresponds to the fraction of tropomyosin molecules moved (Fig. 4). The effect of skS1 is highly cooperative in that each head affects 21–28 actin monomers (in effect the movement of one tropomyosin molecule). This difference can be explained, at least in part, by the fact that both heads of HMM have to bind within the same cooperative unit and thus are less effective than single-headed S1. In such a case, however, the HMM effect should level off at twice the concentration of S1, that is at a head/actin ratio of about 2:7 as opposed to about 1:7 for S1. Since the HMM effect usually levels off at a head/actin ratio between 3:7 and 5:7, the effect of HMM on tropomyosin must be more complex. A similar difference in cooperativity between skS1 and skHMM has been observed by others in the head-induced transition of smTm from off to on states (38, 41).

The calculation of distances from fluorescence resonance energy transfer depends, among other factors, on the relative orientation of the donor and acceptor probes (see Ref. 23 and references to reviews therein). This is expressed in the factor $k^2$, which can be assumed to have a value of ½ if there is
Similarly, in this work we found that FRET between constant during the skS1 titration of FRET of the heterodimer.

The fraction of AED36smTm moved is assumed to equal the fractional change in AED36smTm fluorescence. Myosin heads are as follows: skS1 (closed circles), skHMM (open squares), smHMM-SP (open squares). The curves (solid lines) were generated from a random head binding model with different values for the cooperative unit parameter \( n \), using the equation

\[
\frac{f_m}{1-f_m} = 1 - (1-f_m)^n
\]

where \( f_m \) represents the fraction of AED36smTm moved, \( f_m \) represents the fraction of actin monomers occupied with heads, and \( n \) represents the number of actin monomers affected. 

First of all, the mobility of the AED probe on AED36smTm which remained constant upon titration with smHMM-SP, in-fluorescence polarization, which had a value close to 0.15 and bound to unlabeled actin was assessed by determining the number of actin monomers affected, via the movement of AED36smTm, by the binding of each S1 or HMM molecule to the actin thin filament.

The experimental rigid limit polarization of the parent IAEDANS probe (0.35) is low compared with the theoretical limit of 0.5 (43). This suggests multiple transitions with mixed polarizations, which further reduces the uncertainty in assuming that \( \kappa^2 \) is \( \Omega/2 \) (44). However, more importantly for this study, for changes in FRET upon titration with myosin heads to be interpreted as due to changes in distance between donor and acceptor, the actual value of \( \kappa^2 \) is unimportant, but it is necessary that it does not change. 

In order to evaluate the validity of this assumption, the following experiments were conducted. First of all, the mobility of the AED probe on AED36smTm bound to unlabeled actin was assessed by determining the fluorescence polarization, which had a value close to 0.15 and which remained constant upon titration with smHMM-SP, indicating a lack of change in \( \kappa^2 \) due to a change of mobility of the AED probe. The same results were obtained with skS1 in the previous work (23). In the previous study, we also showed that, although skS1 changed FRET between AED36smTm \( \alpha\beta\)-heterodimer and DAB-A, skS1 had little or no effect on FRET between AED36smTm \( \beta\beta\)-homodimer and DAB-A (Fig. 7 of Ref. 23). This experiment had verified our hypothesis that FRET of the homodimer would be less sensitive than that of the heterodimer to the movement of tropomyosin (23). This finding also served as indirect evidence that \( \kappa^2 \) remained essentially constant during the skS1 titration of FRET of the heterodimer. Similarly, in this work we found that FRET between AED36smTm \( \beta\beta\)-homodimer and DAB-A changed very little, if at all, upon titration with smHMM-SP (data not shown).

However, it is possible that FRET between the AEDsmTm homodimer and DAB-A is relatively insensitive to changes in orientation, mobility, or movement of DAB on actin as it is to the movement of tropomyosin. Furthermore, since DAB is non-fluorescent, its mobility cannot be easily assessed. For these two reasons we developed another set of experiments to test if smHMM-induced changes in DAB on actin could account for the smHMM-induced changes in FRET that we observed. We have found that the disulfide cross-linking of chicken gizzard CaD Cys\textsuperscript{580} to actin Cys\textsuperscript{374} with 50 \( \mu \)M dithiobis(2-nitrobenzoic acid) as revealed by 7\% SDS-PAGE. Lane A, CaD alone before cross-linking showing the presence of small amounts of internally disulfide-cross-linked monomer (M) and disulfide-cross-linked dimer (D) (58). CaD was cross-linked to actin (\( \times \)) in the presence of smTm in the absence of skS1 (lane B) and in the presence of skS1 at skS1/actin ratios of 1:7 (lane C) and 2:7 (lane D). Conditions were as follows: 5.35 \( \mu \)M actin, smTm/actin ratio of 1:7, CaD/actin ratio of 1:15 in 40 mM NaCl, 2 mM MgCl\textsubscript{2}, 10 mM Mops, pH 7.5. Actin and smTm cannot be seen on this gel, since they have run off.

Therefore, we measured FRET between AED-CaD and DAB-A in the presence of tropomyosin as a function of smHMM-SP (Fig. 6). The addition of DAB-A plus smTm to AED-CaD resulted in a drop in fluorescence as a result of energy transfer from AED to DAB. The degree of transfer is somewhat greater than indicated in the figure, since unlabeled actin plus smTm causes a small increase in AED-CaD fluorescence. The addition of smHMM-SP at a head/actin ratio of 1:7 did not result in any further change in fluorescence (Fig. 6) (i.e. in FRET), and AED-CaD (and smHMM-SP) remained 100% bound to the DAB-A plus smTm. Upon the further addition of

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**Fig. 4.** Cooperativity of smooth muscle tropomyosin movement by myosin heads. The fraction of AED36smTm moved is assumed to equal the fractional change in AED36smTm fluorescence. Myosin heads are as follows: skS1 (closed circles), skHMM (open squares), smHMM-SP (open squares). The curves (solid lines) were generated from a random head binding model with different values for the cooperative unit parameter \( n \), using the equation

\[
\frac{f_m}{1-f_m} = 1 - (1-f_m)^n
\]

where \( f_m \) represents the fraction of AED36smTm moved, \( f_m \) represents the fraction of actin monomers occupied with heads, and \( n \) represents the number of actin monomers affected.

**Fig. 5.** Disulfide cross-linking of chicken gizzard CaD Cys\textsuperscript{580} to actin Cys\textsuperscript{374} with 50 \( \mu \)M dithiobis(2-nitrobenzoic acid) as revealed by 7\% SDS-PAGE. Lane A, CaD alone before cross-linking showing the presence of small amounts of internally disulfide-cross-linked monomer (M) and disulfide-cross-linked dimer (D) (58). CaD was cross-linked to actin (\( \times \)) in the presence of smTm in the absence of skS1 (lane B) and in the presence of skS1 at skS1/actin ratios of 1:7 (lane C) and 2:7 (lane D). Conditions were as follows: 5.35 \( \mu \)M actin, smTm/actin ratio of 1:7, CaD/actin ratio of 1:15 in 40 mM NaCl, 2 mM MgCl\textsubscript{2}, 10 mM Mops, pH 7.5. Actin and smTm cannot be seen on this gel, since they have run off.
smHMM-SP up to a head/actin ratio of 2:7, there was only a slight increase (~5%) in fluorescence due to a drop in AED-CaD binding to DAB-A plus smTm to 95% of maximum. At higher concentrations of smHMM-SP, the binding of AED-CaD started to drop precipitously, and the fluorescence increased. Thus, FRET was essentially constant up to a HMM head/actin ratio of 2:7, whereas, under these same conditions, FRET between AED36smTm and DAB-A decreased dramatically (Fig. 2). Therefore, we conclude that 1) smHMM-SP binding to actin did not move CaD, consistent with the cross-linking results and 2) smHMM-SP did not induce any change in the DAB acceptor on actin, which could account for the change in FRET between AED36smTm and DAB-A.

**DISCUSSION**

The interpretation that changes in FRET by smHMM-SP are due to changes in distance between donor and acceptor probes and thus to the movement of tropomyosin depends on the assumption that the orientation factor $k^2$ remains constant (see above). Several observations suggest that $k^2$ is indeed constant in the experiments reported in this work. First of all, smHMM-SP had no effect on the AED polarization of AED36smTm bound to DAB-A, indicating that the binding of smHMM-SP to the thin filament did not restrict the relatively high mobility of the AED probe on smTm. Furthermore, smHMM-SP had no effect on the fluorescence intensity of AED36smTm bound to unlabeled actin (see “Experimental Procedures”), suggesting that during titration with smHMM-SP the AED probe’s environment and/or solvent accessibility did not change. This is consistent with the smHMM-SP having no effect on the orientation or mobility of the AED probe of AED36smTm bound to DAB-A. Since DAB is nonfluorescent, a similar direct analysis cannot be conducted on DAB-A. However, the fact that smHMM-SP does not substantially change FRET between AED36smTm homodimer and DAB-A or between AED-CaD (which does not move) and DAB-A is strong evidence that changes in FRET between the AED36smTm heterodimer and DAB-A are not due to changes in orientation of the DAB probe. This conclusion is consistent with an ESR study of a spin label attached to Cys374 of actin, which showed that the orientation distribution of the spin label did not change significantly upon the binding of myosin heads to actin (46). Thus, although we cannot entirely rule out the possibility that changes in FRET are due to changes in $k^2$, taken all together, these observations strongly suggest that the changes in FRET of the AED36smTm/DAB-A system with the addition of smHMMSs are not due to changes in $k^2$ but to changes in distance between AED and DAB due to a movement of tropomyosin.

The movement of tropomyosin by smooth muscle myosin heads is clearly controlled by the phosphorylation of heads. In the absence of nucleotide (i.e., in rigor), it takes considerably more smHMM to move tropomyosin than it does for smHMM-SP. In the presence of ADP, the difference is greatly emphasized in that it is absolutely necessary for heads to be phosphorylated to induce movement of tropomyosin. This latter situation would appear to be more relevant to the head-induced movement of smTm in vivo, since, in the live muscle, myosin with bound ADP would be much more prevalent than rigor heads. In contrast, the movement of tropomyosin by skeletal muscle myosin heads appears to be the same regardless of the presence of ADP (23). This difference could be related to the finding that ADP binds more strongly to smooth muscle myosin than to the skeletal muscle isoform (47, 48), resulting in a large ADP-induced structural change in smooth muscle myosin but not in skeletal myosin (49, 50).

Smooth muscle tropomyosin enhances (or potentiates) the actin-activated ATPase activity of phosphorylated smooth muscle myosin by some unknown mechanism (see Introduction). It appears that it is necessary to have a certain minimum myosin head/actin ratio to accomplish significant potentiation, since below this ratio the tropomyosin has very little effect or it inhibits the ATPase activity, depending on the ionic strength (12, 51, 52). The potentiation increases cooperatively with increasing active head or inactive rigor head concentration and eventually levels off. Thus, the binding of myosin heads to actin switches the tropomyosin from a low potentiating or inhibiting ATPase activity state to a high potentiating ATPase activity state, and the degree of potentiation is dependent on the head/actin ratio. Presumably, in vivo the level of contractile force would increase with the level of potentiation. We propose that the head-induced switching of tropomyosin from the low potentiating or inhibiting state to the high potentiating state corresponds to the head-induced movement of tropomyosin to a site on actin that affords potentiation and that the myosin heads must be phosphorylated in order to induce such a movement. In such a manner, tropomyosin movement would act in concert with myosin phosphorylation in switching on/off or in modulating smooth muscle contraction. However, changes in tropomyosin position on the thin filament cannot fully explain the activation of smooth muscle myosin by phosphorylation, which activates actin-myosin ATPase activity even in the absence of tropomyosin.

Smooth muscle, which surrounds the periphery of hollow organs, is able to maintain force over long periods of time at the expense of very little energy, in order to maintain a fixed organ dimension. This highly energy-economic condition has been attributed to a physiological state of smooth muscle fibers called the latch state. Latch arises from fibers that are activated by myosin phosphorylation and then maintain the activated force after the myosin becomes dephosphorylated to low levels and ATPase activity (shortening velocity) is low (53). It has been proposed that the molecular basis of the latch state is the dephosphorylation of actin-bound myosin-ADP, which maintains passive tension for long periods by only slowly detaching from actin (54). We have observed that unphosphorylated myosin heads in the presence of ADP cannot move tropomyosin to the postulated potentiating position. Given the above latch model, it would be highly advantageous for the "latch bridges" to be prevented from moving tropomyosin to the potentiating position, where it could potentiate the activity of the...
low concentrations of phosphorylated myosin, which would not only result in greater energy expenditure by the muscle but might also result in an undesired change in organ shape. Thus, under lachty conditions the position of tropomyosin might help to keep the muscle from generating active force. Furthermore, electron microscopy studies put the position of smooth muscle tropomyosin on actin filaments devoid of other proteins, between the on and off positions of skeletal muscle tropomyosin (55). In this position, smooth muscle tropomyosin may be such that it does not inhibit the attachment of the postulated myosin latch bridges to actin (55).

In conclusion, smooth muscle contraction may be regulated or modulated, not only by factors that affect myosin phosphorylation in the thick filament, but also by factors that might affect the position of tropomyosin on the thin filament or phosphorylated myosin’s ability to move tropomyosin. Such thin filament regulation might contribute to the lack of tight coupling between the level of myosin phosphorylation and contractile force in smooth muscle.

In contrast to the movement of tropomyosin by myosin heads, the binding of myosin heads to actin does not move caldesmon up to a head/actin ratio of about 2:7, as revealed by the lack of change of FRET and disulfide cross-linking between the on and off positions of skeletal muscle tropomyosin (55).

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REFERENCES
1. Levis, P. C., and Gergely, J. (1984) CRC Crit. Rev. Biochem. 16, 235–305
2. Lehrer, S. S. (1994) J. Muscle Res. Cell Motil. 15, 232–236
3. Lehrer, S. S., and Geeves, M. A. (1998) J. Mol. Biol. 277, 1081–1089
4. Lehman, W., Craig, R., and Vibert, P. (1994) Nature 368, 65–67
5. Lehman, W., Vibert, P., Uman, P., and Craig, R. (1995) J. Mol. Biol. 251, 191–196
6. Vibert, P., Craig, R., and Lehman, W. (1997) J. Mol. Biol. 266, 8–14
7. Xu, C., Craig, R., Tobacman, L., Harewitz, R., and Lehman, W. (1999) Biophys. J. 77, 985–992
8. Kamm, K. E., and Stull, J. T. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 593–620
9. Small, J. V., and Sobieszek, A. (1986) Int. Rev. Cytol. 64, 241–306
10. Marston, S. B. (1982) Prog. Biophys. Mol. Biol. 41, 1–41
11. Small, J. V., and Sobieszek, A. (1983) in Biochemistry of Smooth Muscle (Stephens, N. L., ed) Vol. 1, CRC Press Inc., Boca Raton, FL
12. Crock, S., and Eisenberg, E. (1973) Proc. Natl. Acad. Sci. U.S. 70, 512–516
13. Allen, P. C., and Gergely, J. (1984) J. Cell Biol. 98, 1009–1019
14. Lehman, W., Craig, R., and Vibert, P. (1994) Nature 368, 65–67
15. Lehman, W., Vibert, P., Uman, P., and Craig, R. (1995) J. Mol. Biol. 251, 191–196
16. Vibert, P., Craig, R., and Lehman, W. (1997) J. Mol. Biol. 266, 8–14
17. Xu, C., Craig, R., Tobacman, L., Harewitz, R., and Lehman, W. (1999) Biophys. J. 77, 985–992
18. Kamm, K. E., and Stull, J. T. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 593–620
19. Small, J. V., and Sobieszek, A. (1986) Int. Rev. Cytol. 64, 241–306
20. Marston, S. B. (1982) Prog. Biophys. Mol. Biol. 41, 1–41
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