Movement of Smooth Muscle Tropomyosin by Myosin Heads†

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ABSTRACT: It has been proposed that during the activation of muscle contraction the initial binding of myosin heads to the actin thin filament contributes to switching on the thin filament and that this might involve the movement of actin-bound tropomyosin. The movement of smooth muscle tropomyosin on actin was investigated in this work by measuring the change in distance between specific residues on tropomyosin and actin by fluorescence resonance energy transfer (FRET) as a function of myosin head binding to actin. An energy transfer acceptor was attached to Cys374 of actin and a donor to the tropomyosin heterodimer at either Cys36 of the β-chain or Cys190 of the α-chain. FRET changed for the donor at both positions of tropomyosin upon addition of skeletal or smooth muscle myosin heads, indicating a movement of the whole tropomyosin molecule. The changes in FRET were hyperbolic and saturated at about one head per seven actin subunits, indicating that each head cooperatively affects several tropomyosin molecules, presumably via tropomyosin's end-to-end interaction. ATP, which dissociates myosin from actin, completely reversed the changes in FRET induced by heads, whereas in the presence of ADP the effect of heads was the same as in its absence. The results indicate that myosin with and without ADP, intermediates in the myosin ATPase hydrolytic pathway, are effective regulators of tropomyosin position, which might play a role in the regulation of smooth muscle contraction.

Muscle contraction is initiated by the infusion into the muscle cell of Ca^{2+}, which allows myosin heads from the thick filament to cyclically interact with actin in the thin filament such that the filaments slide past each other and contraction ensues. The hydrolysis of ATP by myosin, which is activated by its interaction with actin, provides the energy for this process. Regulation of contraction is thought to involve tropomyosin, an extended coiled-coil dimer, which binds end-to-end along the actin thin filament of muscle, each molecule spanning seven actin monomers. In skeletal muscle, in the absence of Ca^{2+}, the tropomyosin conformation/configuration is such that the actomyosin ATPase activity is low and contraction is essentially shut down. Ca^{2+}-binding to the troponin complex, which binds to each tropomyosin molecule, relieves this inhibition and contraction follows {for review, see ref *3*}. In smooth muscle, calcium activates actomyosin ATPase activity and contraction by binding to calmodulin which, in turn, activates myosin light chain kinase to phosphorylate myosin {for review, see ref *4*}. It is not entirely clear what the role of tropomyosin is in regulating smooth muscle contraction since troponin is not present and the main switch appears to be phosphorylation of myosin in the thick filament.

The binding to actin of strong-binding myosin heads, i.e., in the presence of ADP or in the absence of nucleotide, also appears to take part in the switching on of actomyosin ATPase activity. This switching is cooperative and is thought to be due to a change in the state of tropomyosin {for review, see refs *5*−*8*}. The cooperativity is greater in the presence of smooth muscle tropomyosin than with skeletal muscle tropomyosin (*9*), and the greater end-to-end interaction of the former may be responsible for this difference (*10*). In general, it is thought that tropomyosin propagates switching signals along the thin filament via its binding to seven actin monomers and by its end-to-end interaction, thus affecting a highly cooperative switching mechanism. Strong-binding heads also appear to activate force in skeletal (*11*) and smooth (*12*) muscle fibers, further suggesting that the binding of strong-binding myosin heads to actin is necessary for muscle contraction to be fully switched on.

There is some indication that the change in state of tropomyosin induced by Ca^{2+} and/or myosin heads involves the movement of tropomyosin. X-ray studies of intact muscles have suggested that during muscle activation the position of tropomyosin changes from one which sterically blocks the myosin interaction site on actin to one which does not (*13*−*17*). This simple steric blocking model of regulation has since been revised to one in which tropomyosin only partially blocks or hinders the actin−myosin interaction in the off state (*18, 19*) and that this steric hindrance takes place after the binding of myosin to actin (*20, 21*).

Since the interpretation of X-ray data as indicating a movement of tropomyosin is not unambiguous (*22, 23*), 3-D reconstruction of electron micrographs of thin filaments has also been used to probe this proposed movement. Lehman et al. (*22, 24*) have concluded from this approach that the tropomyosin position, in skeletal muscle native thin filaments, is different in the presence (on state) and absence (off state)

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of Ca^{2+}. Furthermore the tropomyosin contact site on actin in the off state corresponds to the site thought to be involved in the binding of strong-binding myosin heads, thus supporting a steric blocking mechanism of regulation. Vibert et al. (*25*) have more recently concluded that, in the presence of Ca^{2+}, the binding of myosin heads to actin results in a further movement of tropomyosin to fully expose the myosin binding site on actin, supporting the notion that both Ca^{2+} and myosin binding to thin filaments are necessary to fully switch on the skeletal muscle thin filament.

The electron microscopic studies use actin filaments which are fixed, dehydrated, and stained, procedures which might distort the molecular arrangement of the filament and which result in the loss of the dynamic nature of a protein assembly in solution. Attempts to measure the movement of skeletal muscle tropomyosin on actin in solution have been carried out using fluorescence resonance energy transfer (FRET[1]) between a fluorescence donor attached to tropomyosin and a fluorescence acceptor on actin. Since these probes are attached to proteins specifically, spectroscopic changes can be unambiguously assigned to the labeled protein(s). A movement of tropomyosin would be expected to result in a change in distance between donor and acceptor and thus a change in energy transfer. From a lack of change in FRET between skeletal muscle tropomyosin and actin in the presence of troponin upon addition of Ca^{2+} (*26, 27*), it was concluded that Ca^{2+}-binding to troponin did not result in the movement of tropomyosin (*27*), in constrast to the conclusions of X-ray and electron microscopy studies. However, it has also been suggested that the position of skeletal muscle tropomyosin on actin may be such that its azimuthal movement does not result in a change in distance between the donor on tropomyosin and the acceptor on cys374 of actin (*26, 28*). That is, the movement is "invisible" by FRET.

Since smooth muscle tropomyosin appears to assume a different position on actin (compared to that of the skeletal isoform) (*22, 29*), a position which might make its movement visible by FRET, we used FRET to probe the possible movement of smooth muscle tropomyosin as affected by the binding of myosin heads to actin. This study was further motivated by our ability to donor-label both halves of smooth muscle tropomyosin heterodimer, the native form of the protein, by techniques that we have developed (*30*−*32*). In this way we could monitor the changes in FRET, and thus the movement, of the "whole" tropomyosin molecule. We concluded that myosin head binding to actin resulted in the cooperative movement of smooth muscle tropomyosin, suggesting that such a movement may be involved in the regulation of smooth muscle contraction.

[1] Abbreviations used: FRET, fluorescence resonance energy transfer; skS1 or skHMM, single- or double-headed, respectively, chymotryptic fragments of skeletal muscle myosin; smS1, single-headed papain fragment of gizzard smooth muscle myosin; Mops, 3-(*N*-morpholino)-propanesulfonic acid; IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)-amino)naphthalene-1-sulfonic acid; DAB-mal, *N*(4-((4-(dimethyl-amino)phenyl)azo)-phenyl)maleimide; smTm, gizzard smooth muscle tropomyosin; skTm, skeletal muscle tropomyosin; AED-Tm, tropomyosin labeled with IAEDANS, in general; AED36smTm or AED190smTm, smTm $\alpha\beta$ heterodimer singly labeled at either Cys36 of the β-chain or Cys190 of the α-chain, respectively, with IAEDANS; AED190skTm, skTm $\alpha\alpha$ homodimer labeled at Cys190 with IAEDANS; DAB-A, actin labeled at Cys374 with DAB-mal; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TN, troponin.

EXPERIMENTAL PRODCEDURES

Protein Preparation and Concentration Determination. Myosin from rabbit skeletal muscle was prepared according to Nauss et al. (*33*) and its single-, skS1, and double-, skHMM, headed fragments were prepared by chymotryptic digestion according to Weeds and Pope (*34*). Myosin, S1, and HMM concentrations were obtained from the absorbance at 280 nm using the extinction coefficients $A^{1\%}_{280nm} = 5.6$, 7.5, and 6.47 cm^{-1} (*34*), respectively. Smooth muscle myosin was prepared from chicken gizzard according to Ikebe et al. (*35*) and its single-headed fragment, smS1, by papain digestion according to Greene et al. (*36*). smS1 concentration was determined using an extinction coefficient $A^{1\%}_{280nm} = 7.3$ cm^{-1} (*36*). Actin was prepared from rabbit skeletal muscle according to Spudich and Watt (*37*) and its concentration determined from its absorption at 290 nm using an extinction coefficient $A^{1\%}_{290nm} = 6.3$ cm^{-1} (*38*). Troponin was prepared according to Greaser and Gergely (*39*) and was the generous gift of Dr. Terence Tao. Troponin concentration was determined from the absorbance at 280 nm using the extinction coefficient $A^{1\%}_{280nm} = 4.5$ cm^{-1} (*40*). Skeletal muscle tropomyosin was prepared from rabbit skeletal muscle according to Greaser and Gergely (*41*) and its concentration determined from its absorption at 277 nm using an extinction coefficient $A^{1\%}_{277nm} = 2.4$ cm^{-1} (*42*). Skeletal tropomyosin was separated into its $\alpha\alpha$ and $\alpha\beta$ component dimers by chromatography on hydroxylapatite (DNA grade, Bio-Rad) according to Eisenberg and Kielley (*43*). Smooth muscle tropomyosin was prepared from chicken gizzard (*44*) and further purified by hydroxylapatite chromatography (*43*). The concentration of gizzard tropomyosin was determined with the Lowry assay (*45*) using rabbit skeletal tropomyosin as a standard.

Actin Labeling. Actin was labeled at Cys374 with DAB-mal (Molecular Probes) by reacting globular, monomeric G-actin in G-buffer (2 mM Mops, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.01% NaN_3, pH 7.5) with 2 mol of DAB-mal (in dimethylformamide)/mol of actin overnight with stirring at 4 °C according to Tao et al. (*26*). The unreacted label was removed first by sedimenting out the undissolved label (and any possible actin aggregates) and then by exhaustive dialysis against G-buffer after addition of dithiothreitol to react with any remaining unreacted label. The degree of labeling was determined from the concentration of label, determined from the absorption at 460 nm using an extinction coefficient ϵ_{460} nm (M^{-1} cm^{-1}) = 24800 (*26*) and a protein concentration by the Lowry method using unlabeled G-actin as a standard. The labeling ratio was about 0.7. The labeled G-actin was then converted to filamentous F-actin by adding NaCl to 40 mM and $MgCl_2$ to 2 mM. The labeled F-actin will be referred to as DAB-A.

Tropomyosin Labeling. Chicken gizzard smooth muscle tropomyosin is a heterodimer of an α and a β chain[2] (*31*, *46*), with the α chain containing a single cysteine at position 190 (*1*) and the β chain a single cysteine at position 36 (*47*, *48*). The following protocol was used to label the heterodimer with IAEDANS at either Cys36 or Cys190. The heterodimer

[2] In our previous works we have referred to the gizzard tropomyosin α-chain as the γ-chain (*1*). However this chain is now appropriately designated as the α-chain, based on protein and gene sequencing (*2*).

was dissociated in 4 M guanidine HCl, then reassociated to form a 1:1 mixture of the αα and ββ homodimers, by dialysis against 1 M NaCl (*30*), which were separated by ion-exchange chromatography on a Mono-Q column (Pharmacia) (*31*). The ββ homodimer was labeled at Cys36 and the αα homodimer at Cys190 by reacting with 20 mol of IAEDANS/mol of dimer in 4 M guanidine HCl, 3 mM Mops, 0.1 mM EDTA, pH 7.5 for overnight at room temperature. To remove unreacted label, dithiothreitol was first added to react with the label and the labeled homodimers were then exhaustively dialyzed against 40 mM NaCl, 5 mM Mops, 0.2 mM EDTA, 0.01% NaN_3, pH 7.5. The labeling ratios were determined from the protein concentration, measured as described above, and the label concentration from the absorption at 337 nm using an extinction coefficient ϵ_{337nm} (M^{-1} cm^{-1}) = 6100 (*49*). The labeling ratios were between 1.5 and 1.7 mol of label/mol of dimer. To prepare the heterodimer singly labeled at Cys36 (AED36smTm), the Cys36-labeled ββ homodimer was combined with unlabeled αα homodimer at an αα/ββ molar ratio = 1.15, incubated at 50 °C to dissociate all dimers into single chains, and then reassociated as heterodimer by slowly cooling (*32*). Heterodimer singly labeled at Cys190 (AED190smTm) was similarly prepared.

Skeletal muscle αα tropomyosin was labeled at its single cysteine at position 190 by reacting with 20 mol of IAEDANS/mol of dimer in 4 M guanidine HCl, 3 mM Mops, 0.1 mM EDTA, pH 7.5 for overnight at room temperature and then dialyzed against 40 mM NaCl, 5 mM Mops, 0.2 mM EDTA, 0.01% NaN_3, pH 7.5. This labeled tropomyosin will be referred to as AED190skTm.

Fluorescence. Steady state fluorescence and fluorescence polarization spectra of AED-labeled tropomyosin were measured on a Spex Fluorolog 2/2/2 photon counting fluorometer at an excitation wavelength of 340 nm, as described previously (*50*).

FRET between an AED donor attached to tropomyosin (AED-Tm) and a DAB acceptor attached to actin (DAB-A) was measured from the change in steady state fluorescence of the donor label, essentially as we have performed previously (*51*). The spectra of AEDTm, at about 0.6 μM, was measured in the absence and presence of DAB-A, at about 4.8 μM, in a solution containing 25−40 mM NaCl, 5 mM $MgCl_2$, 5 mM Mops, pH 7.5 at 20 °C. The equation defining energy transfer effeciency (E) is $E = 1 - f/f_o$, where f_o is the fluorescence intensity (at 500 nm) of AEDTm in the absence of DAB-A and f is the fluorescence in the presence of DAB-A ± S1 or HMM. It is necessary to correct the energy transfer efficiency for incomplete (i.e., 70%) labeling of actin, i.e., $E_{corrected} = E_{observed}/0.7$ (Fairclough and Cantor, 1978). The distance between donor and acceptor, R, was calculated from the equation $R = R_o(E^{-1} - 1)^{1/6}$, where R_o is the critical transfer distance, i.e., the distance between a particular donor/acceptor pair where the energy transfer effeciency is 50%. $R_o = (8.79 \times 10^{-5} J Q_D n^{-4} \kappa^2)^{1/6}$, where J is the normalized spectral overlap of the donor emission and the acceptor absorption, Q_D is the quantum yield for the donor emission, n is the index of refraction, and κ^2 is a factor which depends on the relative orientation of the donor and acceptor {reviewed in refs *52*−*58*}. If there is sufficient motion of the acceptor and/or donor then $\kappa^2 = 2/3$ (see above reviews). The value of R_o for the AED-Tm/DAB-A pair is taken to be 40 Å, the value calculated for AED attached to Cys190 of αα skeletal tropomyosin and DAB attached to Cys374 of actin by Tao et al. (*26*). They have also demonstrated that, for the AED-skTm/DAB-A pair, $\kappa^2 = 2/3$ is a reasonable approximation (*26*). It is assumed that this value of R_o = 40 Å approximates that for the AED donor attached to Cys36 and Cys190 of the smooth muscle tropomyosin. This value cannot be too far off since the value of R_o for the AED/DAB pair has taken on a rather limited range of values, 39−44 Å, for a variety of biochemical systems (*55*). Furthermore the absolute value of R_o is not critical since the main concern of this work is the change in distance between donor and acceptor.

In FRET experiments it is necessary to control for changes in donor fluorescence which might not be due to energy transfer, before the energy transfer efficiency can be calculated. A correction, roughly 6% under our conditions, was made to f for trivial absorption (*59*) of the excitation and emission light due to the absorption and light scattering by labeled actin. This correction was determined from the optical density at the exciting (340 nm) and emitting (500 nm) wavelength of unlabeled tropomyosin upon addition of DAB-A. It is necessary to correct for changes in AED-Tm fluorescence due to interaction with actin. However addition of unlabeled actin to the various AED-Tms showed no change in fluorescence apart from that due to dilution due to the small volume of actin added. Finally, it is necessary to correct for changes in donor fluorescence due to added myosin heads which might directly affect the fluorescence and which might reduce the fluorescence by trivial absorption due to increased light scattering upon the binding of heads to actin. The correction for these effects turned out to be either very small or negligible. Corrections due to dilution by added myosin heads were also very small. The small corrections described here have been incorporated into the fluoresecence spectra presented in the figures to follow.

Actin Binding. The amount of tropomyosin or myosin heads bound to actin was determined by sedimenting the actin in a Beckman TL-100 centrifuge for 30 min at 80K rpm at 20 °C. The pellet and supernatant were dissolved in SDS−PAGE sample buffer and run on 10% SDS−PAGE according to Laemmli (*60*) with 2 mM $CaCl_2$ included in the running gel in order to resolve both smooth muscle tropomyosin bands from actin (*61*). Tropomyosin and myosin head standards were also run. Gels were scanned into a computer using a UMAX Supervista S-12 scanner and then analyzed using the NIH Image program.

RESULTS

Chicken gizzard smooth muscle tropomyosin is a heterodimer of an α and a β chain[2] (*31*, *46*), with the α chain containing a single cysteine at position 190 (*1*) and the β chain a single cysteine at position 36 (*47*, *48*), both chains containing 284 residues. We have developed methods {(*30*−*32*); see Experimental Procedures)} which allowed us to attach an AED fluorescence donor to either Cys36 or Cys190 of the heterodimer, referred to as AED36smTm or AED190smTm, respectively. This enabled us to measure the change in FRET between either half of the tropomyosin molecule and a DAB acceptor at Cys374 of actin (DAB-A), as a function of single- (S1) and double- (HMM) headed myosin fragments. The AED/DAB pair was chosen since it

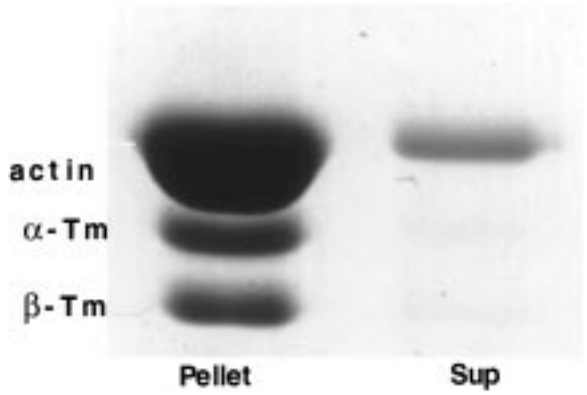

FIGURE 1: Binding of donor-labeled smooth muscle tropomyosin to acceptor-labeled actin. The figure shows a 10% SDS−PAGE of the supernatant and pellet from the centrifugation, at 20 °C, of a solution of AED36smTM (0.6 μM) + DAB-A (4.8 μM) in 25 mM NaCl, 5 mM $MgCl_2$, 5 mM Mops, pH 7.5. The running gel contains 2 mM $CaCl_2$ in order to resolve the αTm chain and actin.

Table 1: Energy Transfer Efficiency (E) and Distance (R) between AED-Donor on Tropomyosin and DAB−Acceptor on Actin ± skS1, skHMM

tropomyosin	myosin head	$E^*_{corrected}$[a]	R[a] (Å)	ΔR (Å)
AED36smTm	−	0.4	43	
	skS1	0.6	37	−6
	skHMM	0.6	37	−6
AED190smTm	−	0.56	38	
	skS1	0.4	43	+5
AED190skTm + troponin	−	0.465	41	

[a] Calculated as detailed in Experimental Procedures, assuming energy transfer is between a single donor and a single acceptor.

had been used previously for FRET between skeletal tropomyosin and actin (*26*).

Since myosin increases tropomyosin binding to actin (*62*), our first objective was to find conditions where AED-Tm bound to DAB-A at close to 100% in the absence of myosin. This was necessary since, if the binding was much less than 100%, the addition of myosin heads would result in an increase in apparent energy transfer efficiency due to increased binding of AED-Tm to DAB-A. We found that if NaCl was kept at 25−40 mM and $MgCl_2$ was not less that 5 mM, the binding of AED36smTm and AED190smTm to DAB-A was greater than 97% at 20 °C. At 40 mM NaCl, 5 mM $MgCl_2$, the binding was greater than 97%, and at 25 mM NaCl, 5 mM $MgCl_2$, the binding was greater than 98% (Figure 1).

The fluorescence of AED36smTm and AED190smTm was decreased upon addition of DAB-A (Figures 2 and 3). This decrease in fluorescence is due to FRET from the AED donor on tropomyosin to the DAB acceptor on actin. The energy transfer efficiencies correspond to a distance between donor and acceptor of 43 Å for the donor at Cys36 near the N-terminus of tropomyosin and 38 Å for the donor at Cys190 in the C-terminal half of tropomyosin (Table 1), assuming that energy transfer is between a single donor and single acceptor. These distances are similar to the distance of 41 Å between the same donor at Cys190 of skeletal muscle tropomyosin and DAB-A (*26*).

Upon addition of the single-headed fragment of skeletal myosin, skS1, the AED36smTm fluorescence decreased further (Figure 2), indicating increased FRET to DAB-A,

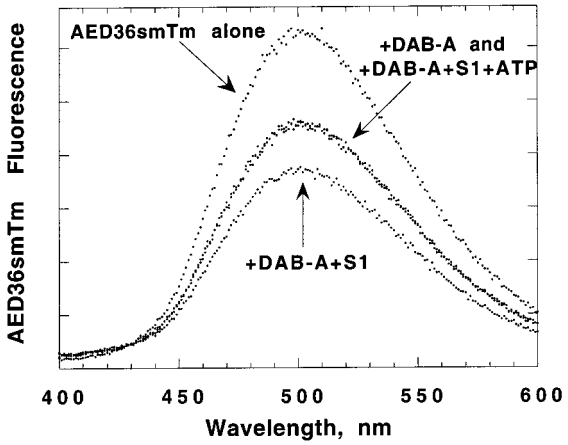

FIGURE 2: Fluorescence spectra of AED36smTm ± DAB-A ± skS1 ± Mg^{2+}ATP. Tm = 0.6 μM; actin = 4.8 μM in 40 mM NaCl, 5 mM $MgCl_2$, 5 mM Mops, pH 7.5 at 20 °C. skS1/actin = 3/7; Mg^{2+}-ATP = 1 mM. The excitation wavelength was 340 nm.

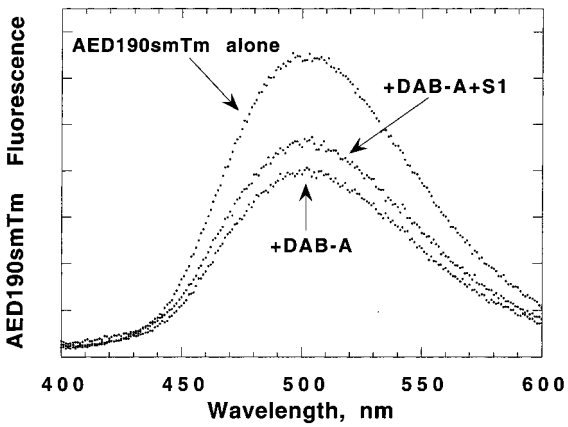

FIGURE 3: Fluorescence spectra of AED190smTm ± DAB-A ± skS1. skS1/actin = 2/7. Other conditions as in Figure 2.

whereas the AED190smTM fluorescence increased (Figure 3), indicating decreased FRET to DAB-A. Both changed hyperbolically and leveled off at an skS1/actin molar ratio of about 1/7 (Figure 4). The same results were obtained at either 25 or 40 mM NaCl. The skS1-induced change in FRET for AED36smTm and AED190smTM corresponded to a change in distance between donor and acceptor of −6 Å and +5 Å, respectively (Table 1). The binding of skS1 to DAB-A + AED-Tm, under the conditions of the fluorescence titration, was measured at several skS1 concentrations up to skS1/actin = 5/7 and found to be greater than 97% at all levels. In the presence of ATP, which dissociates skS1 from actin, the change in fluorescence induced by skS1 was completely reversed (Figure 2). In the presence of ADP, the fluorescence titration with skS1 was the same as in its absence (Figure 4). Thus strong-binding skS1, whether in the absence or presence of ADP, induces the same changes in FRET. If the double-headed fragment of skeletal myosin, skHMM, was substituted for skS1, the change in FRET was the same, except that the change leveled off at a higher head/actin ratio of 2/7−3/7 (Figure 4; Table 1). Changes in FRET due to a direct effect of S1 on AEDsmTm fluorescence or due to trivial absorption from increased light scattering upon the binding of S1 to actin were very low or insignificant (see Experimental Procedures).

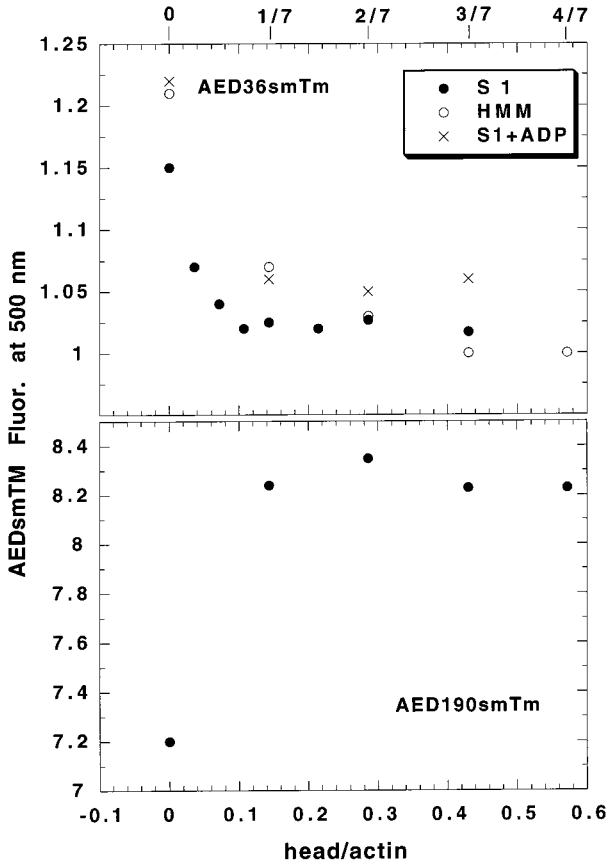

FIGURE 4: Titration of AED36smTm and AED190smTm fluorescence (arbitrary units) in the presence of DAB-A as a function of added skeletal muscle myosin heads: skS1 (closed circles), skS1 +1mM MgADP (×), and skHMM (open circles). Other conditions as in Figure 2.

To assess the relative mobility of the AED donor on tropomyosin bound to actin, the polarization of fluorescence of AED36smTM and AED190smTm, bound to actin, was measured as a function of skS1 up to an skS1/actin molar ratio of 3/7. The polarization of both tropomyosins changed from 0.13 to 0.15 upon binding to actin, and this value did not change upon further addition of skS1 at 20 °C in 40 mM NaCl, 5 mM $MgCl_2$. Thus skS1 binding to actin did not induce any significant change in the mobility of the AED label. This low polarization value compared to the limiting polarization of 0.35 (*49*) for the AED probe indicates that the AED probe has considerable mobility. The same low polarization value was found for AED attached to Cys190 of skTm by Tao et al. (*26*) and thus supported their use of 2/3 as a reasonable value for the κ^2 orientation factor (see Experimental Procedures).

FRET between AEDsmTm and DAB-A was also measured as a function of smooth muscle myosin single-headed fragment, smS1. Although smooth muscle myosin is turned on, i.e., its ATPase activity is activated by actin, only when the myosin heads are phosphorylated, single-headed myosin fragments are constitutively in the on state (*36, 63, 64*). The titration of FRET with smS1 resulted in the same qualitative effects as with the skeletal muscle heads. That is, FRET of AED36smTm decreased and that of AED190smTm increased upon addition of smS1 with the effect leveling off at an smS1/actin ratio of about 1/7 (Figure 5). However the degree of change was smaller with the smS1, which resulted in a

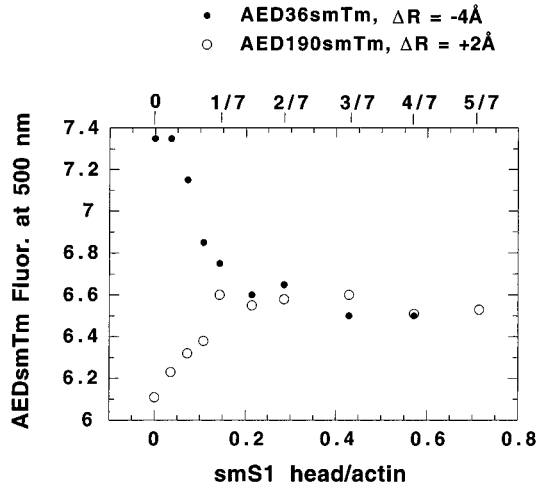

FIGURE 5: Titration of AED36smTm (closed circles) and AED190smTm (open circles) fluorescence (arbitrary units) in the presence of DAB-A as a function of added smooth muscle myosin heads, smS1. Other conditions as in Figure 2.

change in distance between donor on smTm and acceptor at actin Cys374 of −4 Å for the donor at Cys36 and +2 Å for the donor at Cys190 of smTm (compare Figure 5 to Figure 4 and to Table 1).

Since the cooperative response of the state of tropomyosin to the binding of myosin heads is possibly due to tropomyosin's end-to-end interaction, we wished to compare the movement of skeletal muscle tropomyosin to that of smooth muscle since the end-to-end interaction of the latter is much greater than that of the former {(*30, 31*) and refs therein}. However, for skeletal muscle tropomyosin labeled at Cys190, AED190skTm, the binding to DAB-A was considerably less than 100% under the conditions where labeled smooth muscle tropomyosin bound greater than 97%. Thus it was difficult to unambiguously detemine how much of the change in FRET with added S1 was due to bound tropomyosin and how much was due to an apparent change in FRET due to increased binding of AED190skTm to DAB-A. The weaker binding of the labeled skeletal muscle tropomyosin to actin, compared to the smooth muscle isoform, is most likely due to its lower end-to-end interaction, an interaction which is thought to be critical for the strong binding of tropomyosin to actin (*65*). It has also been shown that placing a spectroscopic probe at Cys190 of skeletal muscle tropomyosin reduced its end-to-end interaction considerably (*66*). Furthermore, a considerable fraction of AED190skTm is doubly labeled whereas the smooth muscle tropomyosin molecule has only a single label which would be expected to have less of an effect on the end-to-end interaction.

To increase the binding of AED190skTm to DAB-A, troponin (TN) was added, with the resulting binding being very close to 100% (data not shown). Therefore the addition of TN to AED190skTM + DAB-A resulted in a decrease in AED fluorescence, i.e., an increase in FRET, consistent with the increased binding (Figure 6). Tao et al. (*26*) found a similar increase in FRET upon addition of TN to the same system. And like previous work (*26, 27*), we found that the energy transfer in the presence of TN corresponded to a distance between donor and acceptor of 41 Å (Table 1) and that the addition of Ca^{2+} had no further effect upon FRET (Figure 6). Titration of AED190skTm fluorescence in the

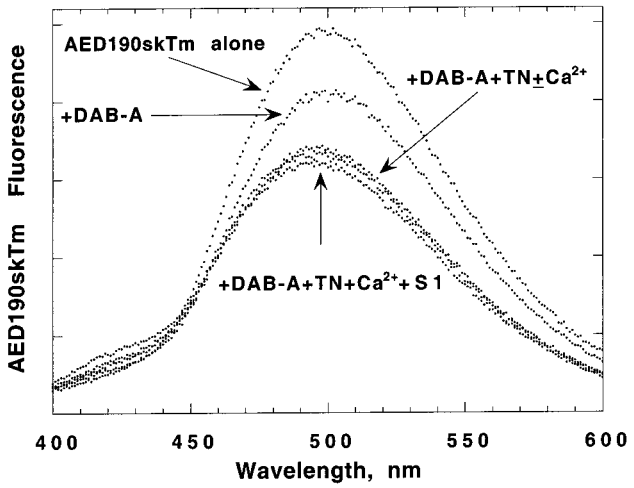

FIGURE 6: Fluorescence spectra of AED190skTm ± DAB-A ± TN ± Ca^{2+} ± skS1. skS1/actin = 2/7. Other conditions as in Figure 2, except that TN = 0.7 uM, EGTA = 0.2 mM in the absence of Ca^{2+}, and $CaCl_2$ = 0.2 mM over EGTA in the presence of Ca^{2+}.

presence of DAB-A +TN +Ca^{2+} with skS1 (Figure 6) or skHMM (data not shown) also resulted in no significant change in fluorescence, thereby not allowing us to correlate the cooperativity of S1-induced Tm movement with Tm end-to-end interaction. In general, we cannot determine whether S1 moves skTm, in the presence or absence of TN.

The FRET studies with smTm were carried out with the singly labeled heterodimer whereas the skTm work {in this and previous studies (*26*, *27*)} was done with the skTm homodimer. We considered the possibility that the skTm homodimer (donor-labeled at either one or both chains) has a donor symmetrically protruding from either side of the Tm coiled-coil and that the donor, in effect, spans a distance of about 40 Å (the length of two donors at about 10 Å and the width of Tm of about 20 Å). Consequently the energy transfer from such a "broad" donor may not have the resolution necessary to clearly see net changes of about 5 Å between donor and acceptor which we have seen for the smTm heterodimer. In this hypothesis it is assumed that the singly labeled Tm heterodimer binds to itself end-to-end on actin such that the labeled chain is always on the same side of the Tm polymer. To test this hypothesis we first labeled the smTm $\beta\beta$ homodimer at Cys36 with AED and measured its FRET to DAB-A as a function of added skS1. {Since the AED/Tm labeling ratio was 1.2, 48% of the Tm was labeled at one chain, 36% was labeled at both chains, and 16% was unlabeled.} Consistent with the hypothesis, skS1 caused little or no change in FRET (Figure 7). We than labeled skTm $\alpha\beta$ heterodimer at either Cys190 of the α chain or Cys36 of the β chain with AED and measured FRET in the presence of DAB-A + TN as a function of Ca^{2+} and skS1 and found that these AEDskTm's also showed no change in FRET with Ca^{2+} or skS1 (data not shown). Thus we conclude that although it might be necessary to use Tm heterodimer labeled at only one of the chains in order to observe Tm movement by energy transfer, it is not sufficient.

DISCUSSION

For donor-labeled smooth muscle tropomyosin bound to acceptor-labeled actin, strong-binding (skeletal or smooth muscle) myosin heads, i.e., in the absence of nucleotide or

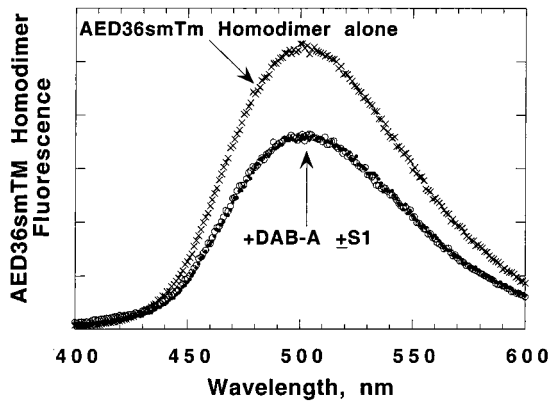

FIGURE 7: Fluorescence spectra of AED36smTm $\beta\beta$ homodimer ± DAB-A ± skS1. X, AED36smTm homodimer alone; closed circles, +DAB-A; open circles, +DAB-A +skS1 at skS1/actin = 2/7. Other conditions as in Figure 2.

in the presence of ADP, resulted in a change in FRET for the donor at either Cys190 in the C-terminal half of tropomyosin or at Cys36 in the N-terminal half of the molecule. These changes in FRET correspond to changes in distance between the smTm cysteines and actin and strongly suggest that myosin head binding to actin results in a movement of the entire tropomyosin molecule on the actin thin filament since: (1) donor probes in both halves of tropomyosin changed their distance relative to actin Cys374; (2) the magnitude of the distance change was about the same for both halves of the molecule; and (3) the dependence of the distance change on myosin head binding to actin leveled off at S1/actin $\sim$ 1/7 for both halves of the tropomyosin molecule. We believe that this is the first clear demonstration of the movement of tropomyosin on actin filaments in solution.

Although we cannot entirely rule out the possibility that the S1-induced changes in FRET are due to changes in probe orientation or mobility, i.e., in κ^2 (see Experimental Procedures), it seems highly unlikely for the following reasons. First of all, myosin heads have little or no effect on the fluorescence intensity (see Experimental Procedures) or fluorescence polarization of the AED donor probe attached to either Cys36 or Cys190 of actin-bound, smTm heterodimer. These results are consistent with the heads having little or no effect on the conformation, mobility, or orientation of the donor probes. Since the acceptor probe, DAB, is not fluorescent, it is not possible to easily check the effect of myosin heads on this probe. However, S1 had no effect on the FRET of AED-labeled skTm homo- or hetero-dimer or smTM $\beta\beta$ homodimer bound to DAB-A. One would have expected to see changes in FRET with these systems if the S1-induced changes in FRET observed for the smTm heterodimer were due to S1-induced changes in the orientation or mobility of the DAB and/or AED probes. Additionally, FRET between AED attached to caldesmon and DAB-A showed no change as a function of added S1 (unpublished results), also supporting the lack of effect of S1 binding to DAB-A on the mobility/orientation of the DAB label. These conclusions are in accord with a study of a spin-label attached to Cys374 of actin which showed no change in orientation distribution of the label upon the binding of S1 to actin (*67*).

The S1-induced movement of tropomyosin resulted in a change in distance between donor and acceptor of 2−6 Å.

Since tropomyosin is thought to move azimuthally on the actin filament, this distance change can be significantly smaller than the actual distance moved by tropomyosin. That the donor probe in the N-terminal half of tropomyosin moves closer to actin Cys374 and the donor probe in the C-terminal half moves farther from actin Cys374 suggests that the Cys36 region of tropomyosin is positioned differently relative to actin Cys374 compared to that of Cys190 relative to Cys374 of a different actin subunit in the actin filament. For example, if Cys36 and Cys190 of tropomyosin were on opposite sides of actin Cys374, relative to the direction of tropomyosin movement, then this would qualitatively explain the observations that we have made. Another possibility is that one or both of the donors on tropomyosin are transferring energy to more than one actin monomer, in which case the change we have measured is due to a net change of distance between donor and more than one acceptor. In any case, our results point to some difference in the spatial relationship between actin Cys374 and the two cysteines of tropomyosin.

For skTm bound to actin we found no effect of S1 or HMM on the energy transfer between Tm and actin. However 3-D electron microscopic reconstructions (*25*) and FRET studies (*68*) have given evidence for the movement of skTm by myosin heads, although in the latter study it was pointed out that the change in FRET could have been due to changes in orientation of the donor (acrylodan) and/or acceptor (intrinsic tryptophans) probes. It is possible that myosin heads do not move skTm on actin filaments in solution but do result in the movement of skTm on actin filaments fixed for microscopic studies. A more likely explanation comes from computer modeling studies of the movement of skTm on actin (*28, 69*) which suggest that a considerable azimuthal movement on actin could go practically undetected, i.e., is "invisible", by FRET. That is, the movement of skTm is such that the distance between the donor on Cys190 and an acceptor on Cys374 of actin remains practically constant. That the movement of smooth muscle tropomyosin is visible by FRET could be due to a different arrangement of smooth muscle tropomyosin on actin as compared to that of the skeletal muscle isoform. Indeed there is evidence from electron microscopy that there is a difference in the position of the two tropomyosins on actin (*22, 29, 70*).

The movement of smooth muscle tropomyosin by S1 heads appears to be cooperative since the full movement is complete at S1/actin = 1/7, well below the saturation of actin by S1 at S1/actin = 7/7. The effectiveness of S1-induced movement can be modeled according to Lehrer et al. (*71*), assuming random binding of S1 to actin monomers in the thin filament and that the binding of more than one S1 head within a cooperative unit has no further effect on the movement of tropomyosin, by using the equation: $f_m = 1 - (1 - f_b)^n$, where f_m is the fraction of tropomyosin molecules moved, f_b is the fraction of actin monomers to which S1 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 molecule to the actin thin filament. Several curves were generated for this equation, using multiples of 7 for n and compared to data from Figure 4, assuming that the fractional change in AED36smTm fluorescence corresponds to the fraction of tropomyosin molecules moved (Figure 8). It can be seen that the data are best fit for $21 < n < 28$. That is, the binding of each S1 to the actin filament can effect the movement of 3−4 tropomyosin molecules, spanning 21−28 actin monomers. Although this is approximate, since the data are scanty and somewhat scattered and because of the assumptions in this model, it is clear that the movement of smooth muscle tropomyosin by myosin heads is highly cooperative. The ability of one S1 to cooperatively move more than one tropomyosin molecule most likely involves tropomyosin's ability to link together via its end-to-end interaction. However, there is evidence that thin filament cooperativity due to myosin head binding can be transmitted directly via actin (*72, 73*). In any case, it is not entirely clear how the tropomyosin moves, i.e., whether it moves as a rigid or a flexible rod (*71*).

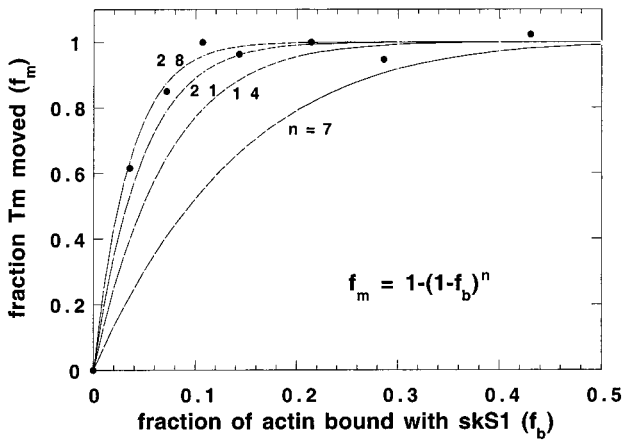

FIGURE 8: Cooperativity of smooth muscle tropomyosin movement by skS1. Comparison of the skS1 titration of the fraction of AED36smTm moved (closed circles), assumed to equal the fractional change in AED36smTm fluorescence from Figure 4, with curves generated from a random skS1 binding model with different values for the cooperative unit parameter n, using the equation $f_m = 1 - (1 - f_b)^n$. f_m = fraction of AED36smTm moved, f_b = fraction of actin monomers saturated with skS1, and n = the number of actin monomers affected, via the movement of AED36smTm, by the binding of each skS1 molecule to the actin thin filament.

These two positions of tropomyosin, i.e., in the absence and presence of myosin heads, most likely correspond to the two states of tropomyosin formulated by Hill et al. (*74*) and extensively characterized by Lehrer, Geeves, and their colleagues (*7, 8, 71*). They have shown that myosin head binding to actin cooperatively switches the tropomyosin between these states which they refer to as either "closed" and "open" or "off" and "on" states. For the S1 dependence of the fluorescence of pyrene-labeled smooth muscle tropomyosin, using the same equation as above, they found for the cooperative unit parameter n a value between 8 and 10 (*71*), a value which is lower than what we found for the movement of smooth muscle tropomyosin by S1. This might be due to a lower end-to-end interaction of their tropomyosin which was the pyrene-maleimide, doubly labeled αα homodimer, whereas we used the singly labeled heterodimer. The homodimer has a lower end-to-end interaction than the heterodimer (*31*) and, since labeling (skeletal) tropomyosin at Cys190 (with pyrene-maleimide) reduces the end-to-end interaction considerably (*66*), double labeling might have a greater deleterious effect than single labeling. The pyrene label may also have a greater effect on reducing the end-to-end interaction than the AED label.

In conclusion, myosin with and without ADP, intermediates in the myosin ATPase hydrolytic pathway, are effective regulators of smooth muscle tropomyosin position. The cooperative movement of tropomyosin by myosin heads might be a key event in the regulation of smooth muscle contraction by a steric blocking mechanism. To more fully understand the role of tropomyosin in smooth muscle regulation it will be necessary to study the effect of unphosphorylated and phosphorylated smooth muscle myosin on the movement of tropomyosin, also in the presence of caldesmon and calponin, other smooth muscle thin filament proteins whose role in regulation is yet to be determined (*75*).

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