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Tropomyosin Exon 6b Is Troponin-specific and Required for Correct Acto-myosin Regulation*

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The specificity of tropomyosin (Tm) exon 6b for interaction with and functioning of troponin (Tn) has been studied using recombinant fibroblast Tm isoforms 5a and 5b. These isoforms differ internally by exons 6a/6b and possess non-muscle exons 1b/9d at the termini, hence they lack the primary TnT_1-tropomyosin interaction, allowing study of exon 6 exchange in isolation from this. Using kinetic techniques to measure regulation of myosin S1 binding to actin and fluorescently labeled Tm to directly measure Tn binding, we show that binding of Tn to both isoforms is similar (0.1–0.5 μM) and both produce well regulated systems. Calcium has little effect on Tn binding to the actin·Tm complex and both exons produce a 3-fold reduction in the S1 binding rate to actin·Tm·Tn in its absence. This confirms previous results that show exon 6 has little influence on Tn affinity to actin·Tm or its ability to fully inhibit the acto-myosin interaction. Thin filaments reconstituted with Tn and Tm5a or skeletal Tm (containing exon 6b) show nearly identical calcium dependence of acto-myosin regulation. However, Tm5b produces a dramatic increase in calcium sensitivity, shifting the activation mid-point by almost an order of magnitude. This shows that exon 6 sequence and, hence, Tm structure in this region have a significant effect upon the calcium regulation of Tn. This finding supports evidence that familial hypertrophic cardiomyopathy mutations occurring adjacent to this region can effect calcium regulation.

Tropomyosins (Tm)[1] are a family of α helical coiled-coil proteins which seem to be ubiquitous among eukaryotic cells, having been identified in organisms ranging from yeast (*Saccharomyces cerevisiae*) to man. They are parallel homo- or heterodimers (encoded from the same or different genes) of two fully α helical chains of identical length, although the length can vary according to isoform type. In mammalian cells, alternative splicing produces a variety of muscle and non-muscle isoforms from four different genes (1). In muscle, the majority of Tm is produced from one α and one β gene product, with a smooth and a striated muscle isoform produced from each gene. In the α gene, exon 6b, which is found in both the muscle isoforms, is replaced by exon 6a in a number of non-muscle isoforms. However the β smooth tropomyosin contains exon 6a rather than 6b. In skeletal muscle, the part of Tm encoded by this sequence is positioned adjacent to the globular "head" region of regulatory troponin complex (Tn) (2, 3). Therefore, it was proposed that there is a muscle Tm-specific interaction with the exon 6 encoded sequence (reviewed in Refs. 4 and 5; also, see Fig. 1). This was argued partly on the basis that the lower coiled-coil stability of exon 6b in comparison to exon 6a (see Fig. 1) was a result of amino acid changes necessary for Tm interaction with Tn, which were unneeded in the non-muscle exon 6a. However, the work of Hammell and Hitchcock-DeGregori (6) showed that exon 6 exchange had little effect upon either the whole Tn complex or TnT affinity for a series of αTm constructs. Therefore, they concluded that exon 6 is not required for interaction with Tn and may instead be important for interactions with caldesmon, as shown by other investigators (7, 8).

To study the effect of αTm exon 6 upon troponin regulation of the acto-myosin interaction, we have used the two αTm fibroblast isoforms Tm5a and Tm5b, which are shown in Fig. 1. These are shorter than muscle tropomyosins because they lack exon 2, but they differ internally only by exon 6. Confusingly, Tm5a has exon 6b, whereas Tm5b possesses exon 6a. They both have a smooth muscle like exon 9d and a non-muscle exon 1b, which, together, have been shown to lack significant interaction with the N-terminal region of TnT (9, 10). This allows the effects of exchange of exon 6 to be studied in isolation of what is regarded as the primary TnT-Tm interaction. As highlighted in Fig. 1, we have also produced a Tm5b mutant, V154C, in exon 6a to give a cysteine at an identical position to Cys^{154} in the exon 6b sequence (equivalent to position 190 in the skeletal sequence). This allows the covalent attachment of a fluorescent pyrene label in the same position as has been previously used for studies of smooth and skeletal muscle tropomyosins (11–14).

Actin thin filaments containing these fibroblast tropomyosins, both in pyrene-labeled and -unlabelled forms, have been characterized for their ability to bind troponin using both a functional kinetic assay based on the regulation of the acto-myosin interaction and by Tn-induced changes in monomer fluorescence of pyrene-labeled Tm on Tn binding. We have measured the effects of calcium upon Tn-saturated thin filaments. The functional assays we used are based upon measurement of occupancy of the biochemical blocked state of the three-state model of thin filament regulation (15). As defined by the work of McKillop and Geeves (15), the blocked state is only significantly occupied in the absence of Ca^{2+} when the Tn complex is tightly bound to the filament. In the presence of Tn and Ca^{2+} or when Tn is absent, K_B (the blocked-closed equilibrium constant) is large, giving little or no occupancy of the blocked state. Hence, measurement of the occupancy of the

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[1] The abbreviations used are: Tm, tropomyosin; Tn, troponin; skTm, skeletal tropomyosin; PIA, pyrene iodoacetamide; PM, pyrene maleimide; EM, electron microscopy; S1, myosin subfragment 1; MOPS, 4-morpholinepropanesulfonic acid; FHC, familial hypertrophic cardiomyopathy.

blocked state can be used both to measure Tn binding to actin·Tm in the absence of calcium and also the calcium dependence of regulation of Tn-saturated thin filaments.

Our measurements show that troponin binding to actin·Tm is not significantly different for exons 6a/6b, which is in agreement with Hamell and Hitchcock-DeGregori (6). However, we show that exon 6 has a large influence on the calcium sensitivity of Tn regulation of thin filaments, with the non-muscle exon 6a increasing the calcium sensitivity by almost an order of magnitude in comparison to either skeletal Tm or Tm5b containing the muscle-like exon 6b. These results are discussed in terms of previous ideas about the muscle specificity and function of exon 6. Our results also have implications for understanding the basis of some of the Tm-based cardiomyopathies.

Some of this work has been presented previously in a preliminary form (16).

EXPERIMENTAL PROCEDURES

Isolation of Proteins from Rabbit Skeletal Muscle—Skeletal Tn and tropomyosin (skTm) along with actin and myosin were isolated from rabbit skeletal muscle. Tn was obtained by using the methods of Greaser and Gergely (17), and skTm was isolated according to Smillie (18). Their concentrations were determined by using $E^{1\%}$ of 4.5 cm^{-1} for Tn and 2.4 cm^{-1} for skTm, with molecular weights of 120 and 65 kDa, respectively. It should be noted that this preparation of native skTm is an $\alpha\alpha/\alpha\beta$ mixture containing around 10% β Tm. Myosin subfragment 1 (S1) was prepared by chymotryptic digestion of rabbit myosin, as described by Weeds and Taylor (19). Its molar concentration was calculated from absorbance measurement at 280 nm using an $E^{1\%}$ of 7.9 cm^{-1} and a molecular weight of 115 kDa. Actin, purified from rabbit skeletal muscle according to Spudich and Watt (20), was labeled at Cys^{374} with *N*-(1-pyrene)iodoacetamide according to Criddle *et al.* (21) and used at a labeling ratio of ~90%. Its molar concentration was determined by its absorbance at 280 nm using an $E^{1\%}$ of 11.08 cm^{-1} and a molecular weight of 40,000 Da. SDS-PAGE of proteins was performed according to Laemmli (22) using 13.5% acrylamide gels followed by staining with Coomassie Blue G-250.

Expression of Recombinant Tropomyosins—Clones of the rat fibroblast tropomyosins 5a/5b were amplified from pET8c (gift from Drs. M. Gimona and D. Helfman) using PCR primers designed to introduce NdeI and BamHI restriction sites for cloning into the bacterial expression vector pJC20 (23). Primer sequences used were 5′-NdeI-GGAATTCCATATGGCGGGTAGCAGCTCGCTGGAG, 5′-BamHI-CGCGGATCCTCACATGTTGTTTAGCTCCAGTAATG. Identical primers were used for both *TPM5a* and *TPM5b*, as they differ only by an internal alternatively spliced exon (Fig. 1). Tm5b-V154C was produced by PCR based site-directed mutagenesis using the pair of primers V154C F 5′-CTCGGAAGGCCAATgTCGACAGCTGGAAGAAC, V154C R 5′-CTTCCAGCTGTCGACGAcATTGGCCTTCCGAGAGC (mutated base shown in lowercase).

For expression, all the clones were transformed into BL21 DE3(pLys) and expressed and purified as described previously (24). In brief, 1 liter of cultures were grown to late exponential phase and induced for 3 h with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested and resuspended in 60 ml of lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 mg/liter DNase, 1 mg/liter RNase) and lysed by passage through a French press (15,000 psi). The majority of *Escherichia coli* proteins were precipitated by heating to 80 °C for 10 min, and the precipitated protein and cell debris was removed by centrifugation. The soluble Tm was then isoelectrically precipitated at pH 4.5 using 0.3 M HCl. The precipitate was pelleted and resuspended in 10–20 ml (depending upon yield) of running buffer (10 mM phosphate, pH 7.0, 100 mM NaCl). This was then further purified using 2 × 5 ml HiTrap Q column (Amersham Biosciences) in tandem and eluted with a 150–400 mM NaCl gradient, with the Tm eluting at around 200–250 mM salt. Fractions were analyzed by SDS-PAGE, pooled, and concentrated by isoelectric precipitation. Extinction coefficients and molecular weights for recombinant proteins were calculated from the sequences using the integrated protein sequence analysis software AnTheProt (Gilbert Deleage, Institut de Biologie et Chimie des Protéines-Centre National de la Recherche Scientifique). Fibroblast 5a/5b protein concentrations were determined by using an extinction coefficient $E_{280\ nm}$ of 4,670 M^{-1} cm^{-1} and molecular weights of 28425.2, 28,566.2, and 28570.0 for 5a, 5b, and 5b-V154C, respectively.

All of the tropomyosins expressed well in *E. coli*, with typical yields

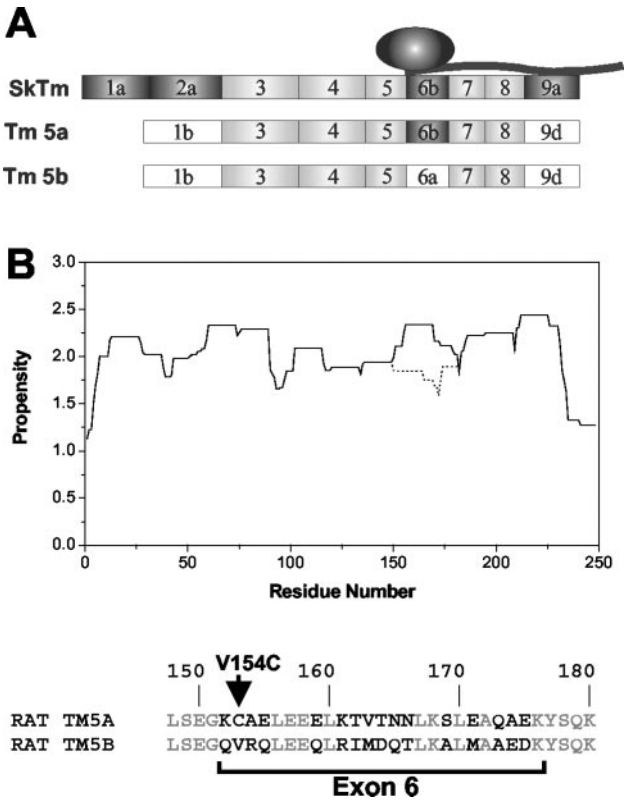

FIG. 1. *A*, exon structure of skeletal α-Tm and the two fibroblast isoforms 5a and 5b with constitutive exons are shown in *light gray*, skeletal exons in *dark gray*, and non-muscle exons in *white*. Exon 6a contains the naturally occurring cysteine found in Tm. The position of the equivalent Val → Cys mutation in exon 6a produced to allow fluorescent labeling is *highlighted* below. *B*, coiled-coil propensity plot of Tm5a/5b was determined according to Lupas (63), showing the lower stability of the skeletal exon 6b in comparison to exon 6a. *Bottom*, sequence alignment of the region containing exon 6 is marked along with the position of the V154C mutation; the non-identical residues are *highlighted*.

of 10–20 mg/liter of culture. The proteins were all expressed as unmodified, unacetylated proteins. Mass spectrometry was used to confirm that the Tms had the correct size and that the N-terminal Met was removed post-translationally to leave Ala as the first amino acid as for the native proteins (25, 26). Small (50 μl) stock samples were dialyzed overnight against 1 mM Tris, pH 7.0, acidified with formic acid, and applied to a Finnegan Mat LCQ ion-trap mass spectrometer fitted with a nano-spray device. Mass accuracy for Tm samples is expected to be 2–4 (1/10,000). Molecular weights of 28425.2, 28,566.2, and 28,570.2 were determined for 5a, 5b, and 5b-V154C respectively, corresponding well to predicted weights for the proteins lacking their N-terminal Met and with no other modification. It has been shown previously that exon 1b in these fibroblast Tms has a five amino acid extension in comparison to exon 1a. This seems to perform the same role in stabilizing the Tm-Tm overlap region (10, 27) as a dipeptide extension commonly used to take the place of the native N-terminal acetylation, which is required for Tms possessing exon 1a to bind to actin with a native-like affinity (28, 29). Tm5a, Tm5b, and Tm5b-V154C all bound to actin in the submicromolar range (data not shown), proving N-terminal modification of the Tm sequence was unnecessary for strong actin binding, as shown previously (30). No evidence was seen of a significant non-binding fraction of expressed protein as reported previously by Pittinger and Helfman for 5a (30).

Kinetic Measurements of S1 Binding to Actin Filaments—Stopped-flow experiments were performed at 20 °C with an SF-61DX spectrophotometer (Hi-Tech Scientific) in fluorescence mode. Pyrene fluorescence of labeled actin was excited at 365 nm and emission was detected at right angles using a KV 389-nm cut-off filter (Schott, Mainz, Germany). Data were stored and analyzed by using the Kinetasyst software provided with the instrument. Transients shown are the average of three to five shots of the machine. In these experiments, the concentrations quoted are those after mixing and are half the relevant concentrations of the stock solutions in the syringes. The occupancy of

the blocked B-state (defined by K_B) has been determined by using the kinetic method of McKillop and Geeves (15), where the rate of binding of S1 to reconstituted filaments is measured under excess actin condition. Under these conditions, the observed rate constant of binding (k_{obs}) is proportional to the fraction of actin available for binding. The value of K_B is then defined from the ratio of the observed rate constant of S1 binding to reconstituted thin filaments in the presence and absence of calcium (15).

$$k_{obs} = [A]k_{+1} \quad \text{and} \quad \frac{-Ca^{2+}k_{obs}}{+Ca^{2+}k_{obs}} \propto \frac{K_B}{1+K_B} \qquad \text{(Eq. 1)}$$

Fluorescence Labeling of Tropomyosins—Cysteine-containing tropomyosins were labeled using pyrene iodoacetamide using a modified method of Ishii and Lehrer (13). Tropomyosins were denatured in a reducing solution of standard buffer containing 6 M urea and 20 mM dithiothreitol at 60 °C for 10 min. The solution was then cooled, and the dithiothreitol was removed by a single-step anion exchange chromatography. The tropomyosin was loaded onto a HiTrap Q column (Amersham Biosciences) and eluted with a single step of 0.4 M sodium chloride. The eluted protein was pooled and rapidly dialyzed against urea containing standard buffer to remove the salt and diluted to 1 mg/ml. Pyrene iodoacetamide stock solution at 100 mg/ml in dimethylformamide was then added at a 10:1 molar ratio and left to stir for 12–18 h. Excess insoluble label was removed by centrifugation at 10,000 × *g* for 10 mins; soluble label and urea were removed by subsequent dialysis against standard buffer. Labeling ratio was calculated spectrophotometrically by calculating the concentration of pyrene from its extinction coefficient at 344 nm of 2.2×10^4 M^{-1} cm^{-1} and correcting the determined absorbance at 280 nm for pyrene absorption using a ratio of 1.104 × $E_{344\ nm}$ to allow calculation of the Tm concentration from its $E_{280\ nm}$ of 4,760 M^{-1} cm^{-1}.

RESULTS

Binding of Troponin to Actin·Tm Filaments—Binding of Tn to actin·Tm filaments reconstituted with the different tropomyosins was measured by using a kinetic assay that measures the occupancy of the blocked B-state produced by Tn binding to and switching off actin·Tm in the absence of calcium.

Fig. 2*A* shows raw kinetic data from a series of stop-flow measurements of myosin binding to actin·Tm filaments reconstituted with increasing quantities of Tn in the absence of calcium. As the Tn concentration was increased, the rate of myosin binding decreased. In terms of the three-state model, this is because of a decrease in the available fraction of actin (Equation 1). Fig. 2*B* shows the secondary plots of k_{obs} *versus* Tn concentration for thin filaments containing tropomyosins 5a, 5b, and 5b-V154C along with skTm as a control. These give a functional assay of Tn binding and are fitted to the quadratic solution of a standard binding equilibrium, with the determined binding constants shown in Table I. As shown by previous works (31–34), Tn binds to actin filaments reconstituted with skTm with a high affinity (<10 nM at these salt concentrations). Binding of Tn to filaments reconstituted with all of the exon 9d containing Tms is >10-fold weaker at 0.27 (±0.068) μM for Tm5a, 0.28 (±0.084) μM for Tm5b, and 0.41 (±0.094) μM for 5b-V154C.

Fluorescence Labeling of Tm—Fig. 3*A* shows the emission spectra of pyrene-labeled Tm5b-V154C at 65% labeling ratio. The spectrum is essentially identical to that of pyrene-labeled Tm5a (not shown) and is similar to that previously measured for pyrene-labeled skeletal and smooth muscle Tms (12, 13), with monomer peaks at 385 and 405 nm and a broad excimer peak at 460 nm.

Fluorescence spectra of Tm5b-V154C showing the effects of sequential addition of actin, troponin to saturation (in the absence of calcium), and then calcium are shown in Fig. 3*B*. A low labeling ratio was used for these experiments to give a reasonable ratio of the measured monomer fluorescence to excimer fluorescence. Excimer fluorescence was produced from Tms in a dimer, both strands having a fluorophor attached; if they are randomly distributed, the statistical fraction carrying

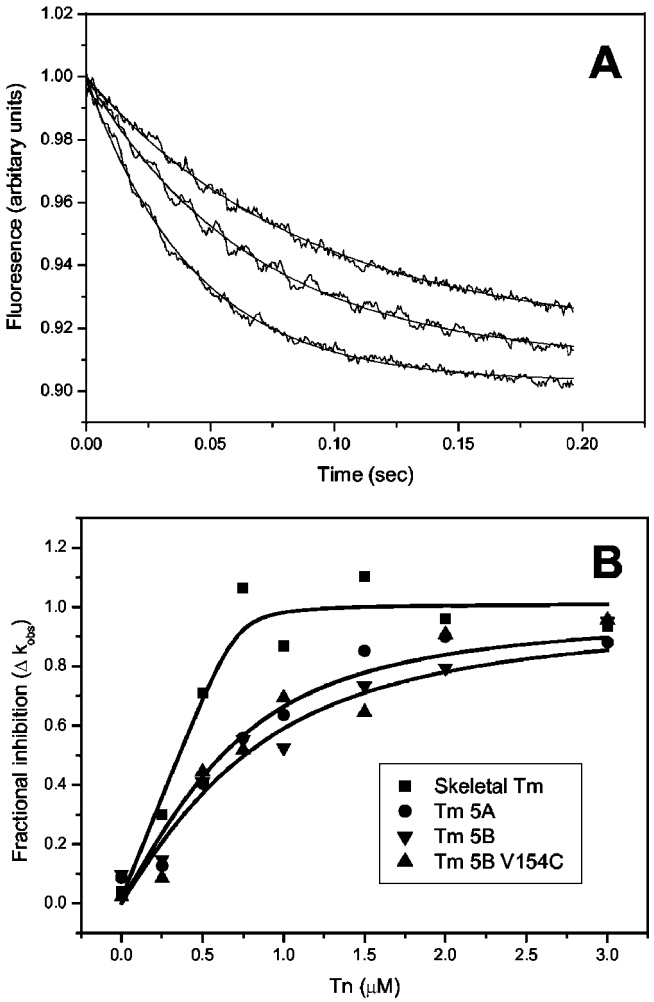

FIG. 2. *A*, representative raw traces with increasing troponin concentration from the kinetic assay were used to measure occupancy of the blocked B-state with single exponential fits (k_{obs}) superimposed. Binding of myosin S1 (0.5 μM) to actin·Tm (5 μM/2 μM) was measured by fluorescence quenching of pyrene-actin in the absence of calcium. As the troponin concentration was increased, the rate of binding decreased. *B*, secondary plot of *A* plotting k_{obs} for S1 binding to thin filaments *versus* Tn concentration. Affinities are determined by fitting to a simple binding model, shown superimposed, with the parameters determined in Table I. The fitted curves for 5a and 5b-V154C are overlapping and have virtually identical values for K_d. Buffer conditions for all experiments were 20 mM MOPS, pH 7, 140 mM NaCl, 5 mM $MgCl_2$, and 1 mM EGTA.

two labels should be equal to the square of the total fraction of Tm-labeled. Thus, at the 30–40% labeling used, this gives a ratio of single- to double-labeled filaments between 1.5–2.33:1, giving a reasonable compromise between absolute signal and proportion of excimer fluorescence.

Initial addition of actin gives a small increase in monomer fluorescence that is in rough proportion to a small decrease in excimer fluorescence. Subsequent addition of Tn produces ~50% enhancement of the monomer fluorescence, with little change in excimer fluorescence. This saturates upon further addition of Tn, suggesting that this signal is caused by Tn binding to actin·Tm and is proportional to the amount of Tn bound. Addition of calcium to Tn-saturated filaments then causes no significant change in signal, showing that the change in fluorescence caused by Tn binding is not calcium sensitive.

Fig. 3*C* shows a secondary plot of fluorescence change at 405 nm, where the largest relative change in monomer fluorescence is found, *versus* concentration of Tn added. This produces binding curves for Tn to the actin·Tm filaments for the two pyrene-labeled tropomyosins Tm5a and Tm5b-V154C. These curves

TABLE I
Parameters determined from curve fits
Errors quoted are standard errors to curve fits. N/A, not applicable; nd, not determined.

	Skeletal Tm	Tm5a	Tm5b	Tm5b-V154C
Tn K_d (μM) (calculated from k_{obs})	<0.01	0.27 ± 0.068	0.28 ± 0.084	0.41 ± 0.094
Tn K_d (μM) (calculated from equilibrium titration)	N/A	0.064 ± 0.007	N/A	0.51 ± 0.10
$pCa_{50\%}$	5.47 ± 0.04	5.50 ± 0.09	nd	6.19 ± 0.16
Hill coefficient	1.73 ± 0.21	1.88 ± 0.69	nd	1.90 ± 1.2

are well fitted by a simple binding equilibrium as for the kinetic binding assays, providing additional evidence that the signal is directly proportional to the Tn bound. Results from the curve fits are shown in Table I, giving Tn affinities of 0.064 (±0.007) μM and 0.54 (±0.10) μM for Tm5a and Tm5b-V154C, respectively.

Calcium Sensitivity of Reconstituted Regulated Filaments—The fraction of Tn with calcium bound can be assayed using basically the same kinetic assay as shown in Fig. 2. The fraction of Tn with calcium bound is directly proportional to the change in k_{obs} for myosin binding to an excess of Tn-saturated thin filaments. Hence, the *p*Ca ($\log_{10}$ [Ca^{2+}]) dependence of k_{obs} can be used as a direct measure of calcium binding to and regulatory switching of troponin.

Fig. 4 shows secondary plots of fractional regulation of filaments *versus* *p*Ca for Tm5a and Tm5b-V154C along with skeletal Tm as a control. The values determined for skTm are similar to those determined previously (35) with a $K_{50\%}$ of 5.47 (±0.04) μM and a Hill coefficient of 1.73 (0.21). Tm5a produces a virtually identical curve with a $K_{50\%}$ of 5.50 (±0.09) μM and Hill coefficient of 1.88 (±0.69). However, Tm5b-V154C produces a significantly different $K_{50\%}$ of 6.19 (±0.16) μM, indicating almost an order of magnitude higher calcium sensitivity but, again, with a similar Hill coefficient of 1.90 (±1.2).

DISCUSSION

The kinetic S1 binding assays show that the troponin has a much lower affinity for thin filaments reconstituted with fibroblast tropomyosins (lacking the skeletal exons 1a/9d) than those with skTm. The importance of the N-terminal TnT-Tm interaction for the high affinity binding of Tn has long been established from the original work of Mak and Smillie (40). This work has recently been supported by a series of CD-based measurements of the stability of complexes formed by peptides containing the N- and C-terminal exon sequences which showed that the N terminus of TnT interacts strongly with the 1a/9a exons found in skeletal Tm but weakly with the 1a/9d combination found in smooth Tm, and not at all with the 1b/9d exons found in the fibroblast Tms used here (10).

The kinetic assays show that replacement of exons 1a/1b and 9a/9d reduces Tn affinity for actin·Tm by >10-fold to 0.2–0.5 μM. This factor of at least 10 is a lower limit, as the binding curve for Tn to skTm·actin is too tight to give anything more than an upper limit for the K_d for Tn binding to actin·skTm. This result is in agreement with previous works that showed a 100-fold reduction in affinity by the replacement of 9a for 9d alone (9, 32). The binding constants for the three proteins, 0.27 (±0.068) μM for Tm5a, 0.41 (±0.094) μM for Tm5b, and 0.28 (±0.084) μM 5b-V154C) are not very different from each other, especially considering the scatter on the data.

The fluorescence-based equilibrium binding data are in general agreement with the kinetic data, showing affinities in a similar range for the PIA-labeled Tms. Our binding curves for Tn to actin·PIA Tm filaments are well fitted by a single-site binding model. In previous work, Ishii and Lehrer (13) fitted their data of Tn binding to pyrene maleimide (PM)-labeled skTm in solution by a two-site model with different affinities for labeled and unlabelled Tm. The necessity for their use of a two-site binding model could be explained by a number of factors, including differences in binding to free Tm (their work) rather than actin·Tm filaments (ours), or the difference in the label used. Tm5a shows some evidence of a small decrease in K_d for binding to actin upon labeling (from 0.27 to 0.064 μM), which is in agreement with the previous data for labels attached to the cysteine in exon 6b, but 5b-V154C shows a small increase from 0.28 to 0.51 μM. These changes are relatively small and inconclusive because of the large scatter on the kinetic data.

The data presented here show that, although lacking the primary N-terminal TnT interaction with Tm, the Tn complex does bind to the fibroblast isoforms with an affinity in the sub-micromolar range, and this does not change significantly upon the addition of calcium. The TnI·TnC complex has a very weak affinity for actin·Tm in the presence of calcium (41–44). Therefore, our data indicate that the measured affinity is due to a secondary interaction specific to the presence of C-terminal TnT "head" region. It had been shown previously that the fluorescence enhancement produced by Tn binding to either isolated PM-labeled skTm (12) or to actin·Tm thin filaments containing PIA Tm (13) was not significantly calcium-dependent at similar near-physiological salt concentrations. However, in both these cases, the Tn was still anchored by the primary N-terminal TnT-Tm interaction, and it had been suggested that the Tm interaction in the TnT head region was weak, especially at higher salt concentrations (12).

Our kinetic data show that exon 6 does not effect the formation of the blocked state, produced by Tn in the absence of calcium, with equal occupancy of the blocked state determined for all of the Tms assayed. This finding is consistent with the work of Hammell and Hitchcock-DeGregori (6), who determined a similar inhibition in ATPase assays for their skTm-like constructs containing either exon 6a or 6b. However, although their biosensor assays suggested little interaction of TnT in isolation with exon 6 or 9d, our binding assays indicate that there is a calcium-independent interaction of the whole troponin complex with the exon 6 region on reconstituted thin filaments.

Significantly, measurement of the calcium dependence of regulation of the acto-myosin interaction shows that there is no difference between the exon 6b-containing skTm and Tm5a, but in contrast, Tm5b containing exon 6a has a 0.7 *p*Ca units higher Ca^{2+} sensitivity. This gives a $K_{50\%}$ of around 600 nM, representing almost an order of magnitude increase in calcium sensitivity. This would result in a much-increased activation in response to calcium transients and would also result in some residual activation even at resting [Ca^{2+}] levels (≥200 nM, *p*Ca 6.7) (45), where the muscle would normally be relaxed. Thus, although we agree with previous work showing that troponin has similar affinities for both exon 6a and 6b (6), our data indicate that the nature of interaction must be significantly different for the different exons, and that the correct exon is critical for proper troponin function.

The exact nature of the differences in the Tm-Tn interactions are unclear. The signal from the PIA fluorophor at position 154

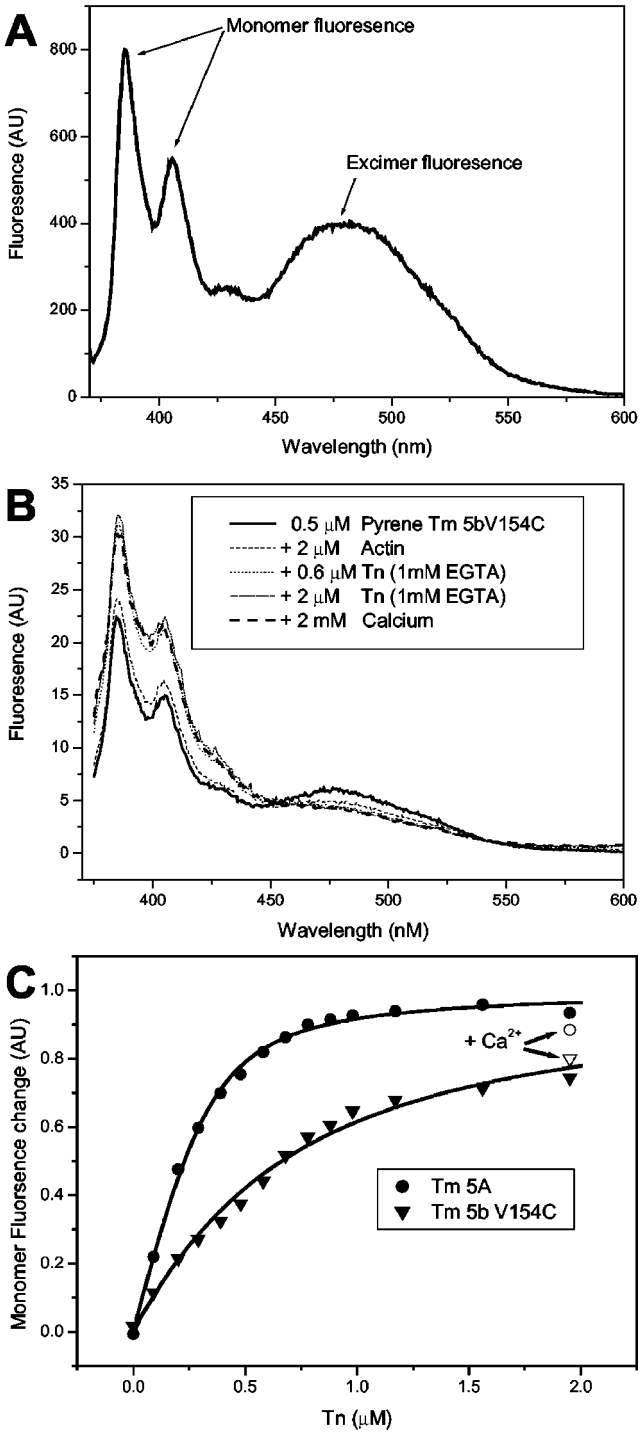

FIG. 3. *A*, the emission spectrum of pyrene-labeled tropomyosin excited at 365 nm showing the two monomer fluorescence peaks centered around 400 nm and the broad excimer emission peak at 460 nm. *B*, effects of the sequential addition of thin filament proteins and then calcium to pyrene-labeled Tm5b-V154C at the concentrations detailed in the figure. *C*, binding curves for the sequential addition of troponin to 5 μM actin and 2 μM pyrene-labeled tropomyosins with buffer concentrations as for Fig. 2. Fluorescence was measured with excitation at 365 nm and emission at the monomer peak was measured at 405 nm. The data were fitted to a simple equilibrium binding model giving the affinities shown in Table I.

(190 in the skeletal sequence) upon Tn binding is quite large but calcium-independent. Thus, Tn binding to actin·Tm causes a substantial change in the environment of the fluorophor, but this is largely unchanged in the calcium-induced regulatory switching of Tn. This occurs despite the effect of exon 6 ex-

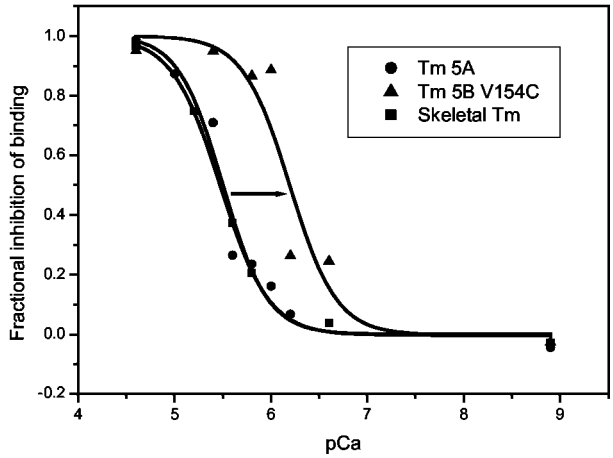

FIG. 4. **Representative secondary plots showing the calcium concentration dependence of regulation of the acto-myosin interaction by Tn in the presence of skeletal Tm, Tm5a, and Tm5b.** Raw data was produced as for Fig. 2*A* by mixing 5 μM actin, 2 μM Tm, 3 μM Tn with 0.5 μM myosin S1 with buffer conditions as described previously. The free calcium concentration was varied by the addition of Ca/EGTA solutions mixed to a final concentration of 2 mM, calculated according to Harrison and Bers (64). The results are plotted as fractional regulation against calcium concentration to give the curves shown. Fractional regulation is defined by comparing the measured rate to the maximum (unregulated) rate when Tn is calcium-saturated to minimum rate when Tn has no calcium bound and is fully inhibitory. Fits to the Hill equation are shown superimposed, with values determined from the fits for the midpoint ($pCa_{50\%}$) and cooperativity (n) shown in Table I.

change upon the affinity of calcium affinity for TnC, which is shown by our data and the large number of structural changes taking place in the actin-TnC-TnI interactions.

Our data, indicating there is no change in the local environment in this region, are compatible with structural data from Lehman *et al.* (39) who visualized a hinge-like movement of troponin in EM reconstructions. Upon binding calcium, the Tn "head" seems to pivot around its Tm contact and away from its actin binding site, with little change in the Tn-Tm contact. Although differing in exact detail, the structures derived using single-particle analysis of EM data by Nartia *et al.* (46) also show that the Tn-Tm contact in the region of the label is maintained in the presence of calcium. The EM data are likely to continue to be of too low a resolution to provide information on the subtle changes in protein-protein interactions responsible for the shift in TnC affinity for calcium seen here. However, the use of further probes at different sites, in combination with the structural data now available for a partial Tn·TIC complex (47), should provide some explanation for these observations. However, there is already some evidence that transmitted structural changes are capable of affecting the calcium affinity of TnC. It has been shown that the calcium-sensitizing drug EMD 57033 binding non-competitively with the inhibitory peptide of TnI to the C-terminal structural domain of TnC increases the calcium sensitivity of cardiac muscle contraction (48). This finding supports the idea that affecting the structural interactions between the regulatory proteins can modulate the overall calcium dependence of regulatory switching.

These results clearly demonstrate that the exon 6 region can affect the calcium sensitivity of regulation of the acto-myosin interaction and may also be significant in understanding the causes of familial hypertrophic cardiomyopathy (FHC) mutations found in tropomyosin. Of the eight FHC mutations currently identified in tropomyosin, four are clustered around exon 6 at positions 175, 180 (×2), and 185 (49, 50). Our data support previous evidence (51) that the FHC mutations found in this region may be affecting calcium regulation. Our data

could also explain the rescue of a *Drosophila* hypercontracting TnI mutant by an S185F Tm mutation, with a similar feed-through effect of Tm structure upon the TnC-calcium-TnI equilibrium (52). However, to produce a rescue of hypercontraction, it would be presumed that the S185F Tm rescue mutation would decrease rather than increase (as seen in the FHC mutations) the calcium sensitivity, allowing the muscle to relax properly.

The observations presented here and almost all of the interpretations of them are independent of the model of acto-myosin regulation used in their interpretation. We report no significant change in affinity of Tn for actin·Tm and no change in the extent to which Tn ($-Ca^{2+}$) slows down the rate of S1 binding to actin for the two Tm isoforms studied. However, we do show that there is a significant difference in the calcium concentration required to relieve the inhibition of S1 binding, depending upon the exon 6 isoform present in Tm. These observations are all model-independent.

We have interpreted the data on the Tn inhibition of S1 binding rates in terms of the McKillop and Geeves three-state model (15, 53) of the thin filament regulatory states (15, 53). However, there are a number of other models of thin filament regulation derived from either solution properties of thin filaments (Tobacman, Ref. 54; Smith, Refs. 55, 56; Chalovich, Refs. 57, 58; or muscle fiber studies, Ref. 59) in combination with data from structural studies of the thin filament (37, 60, 61). The models derived from solution studies are most directly relevant to the work here. All models involve more than a single "off" state of the filament compatible with the EM studies of Lehman and coworkers (37–39). All models also assume that the calcium asserts its influence by means of binding to TnC, which allosterically interferes with the inhibitory interaction between TnI and actin. Loss of the interaction of TnI with actin results in a conformational change in the thin filament, which leads to activation of the interaction of myosin with actin and, hence, the acto-myosin ATPase. Full activation of the thin filament requires the presence of some tightly attached myosin cross bridges. Differences between the models come primarily from the exact description of the different states of the thin filament and the extent and mechanism of the cooperative switching between states. Tobacman (54) invokes actin-actin communication that is propagated along the filament and is linked to the effect of Tm and S1 to the conformation of the actin monomer. The Smith *et al.* (55, 56) model is an extension of the McKillop and Geeves (15, 53) model and deals with Tm strand as a continuous worm-like chain, with the cooperativity being a product of the dynamic flexibility of Tm on the actin surface. The Chalovich *et al.* model (57, 58) remains closest to the original Hill model (62), of which the other models are effectively variants. This has the cooperativity defined in terms of a single A_7Tm cooperativity unit, with additional interaction energy between the A_7Tm units. In fact, the most recent version even appears to have three regulatory states (see figure 1 of Ref. 58).

To date, there is no clear experimental data that distinguish between the different solution-based models of McKillop and Geeves (15, 53), Smith *et al.* (55, 56), Tobacman *et al.* (54), or Chalovich *et al.* (57, 58). In the work presented here, we do not measure any cooperativity parameter or provide data that further defines switching between regulatory states. Hence, this work does not contribute to or clarify the debate among the models. The data measure the calcium sensitivity of the switch between a thin filament which binds S1 slowly to one that binds S1 quickly. This has been shown to give equivalent *p*Ca curves to those produced by measurement of the calcium sensitivity of force generation measured in muscle fibers under similar conditions (reviewed in Gordon *et al.*, Ref. 36). The McKillop and Geeves model (15, 53) has the simplest interpretation of the change of the S1 binding rate to actin in terms of a single parameter (K_B) in the model. The other models do not explicitly address Ca sensitivity in this way, but are, or should be, capable of accounting for such a switch. Speculation about the underlying mechanism for acto-myosin regulation does not detract from the essential results of this work.

In conclusion, our data are in agreement with previous work which has shown that the strength of the Tn-Tm interaction in the exon 6 region does not differ greatly between exons 6a and 6b. Neither is the strength of this interaction significantly calcium-dependent at physiological salt concentrations. However, the variation in the calcium dependence of regulation of the acto-myosin interaction upon sequence shows that the exon 6b sequence has evolved to form a specific troponin interaction that is required for correct troponin functioning.

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