The Sequence of the Alternatively Spliced Sixth Exon of α-Tropomyosin Is Critical for Cooperative Actin Binding but Not for Interaction with Troponin*

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Robin L. Hammell‡ and Sarah E. Hitchcock-DeGregori§
From the Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Tropomyosins, a family of highly conserved coiled-coil actin binding proteins, can differ as a consequence of alternative expression of several exons (Lees-Miller, J., and Helfman, D. (1991) BioEssays 13, 429–437). Exon 6, which encodes residues 189–213 in long, 284-residue tropomyosins, has two alternative forms, exon 6a or 6b, both highly conserved throughout evolution. In α-tropomyosin, exon 6a or 6b is not specific to any one of the nine isoforms. Exon 6b encodes part of a putative Ca2+-sensitive troponin binding site in striated muscle tropomyosins, suggesting that the exon 6-encoded region may be specialized for certain tropomyosin functions.

A series of recombinant, unacetylated tropomyosin exon 6 deletion and substitution mutants and chimeras was expressed in Escherichia coli to determine the requirements of exon 6 for tropomyosin function. Functional properties of the tropomyosins were defined by actin affinity measured by cosedimentation, troponin T affinity using a newly developed biosensor assay, and regulation of the actomyosin MgATPase. The region of tropomyosin encoded by exon 6 affects actin affinity but not thin filament assembly, troponin T binding, or regulation with troponin. The tropomyosins with exon 6a or 6b function normally whether a striated muscle exon 9a or smooth/non-muscle exon 9d is present. However, the effect of deleting 21 amino acids encoded by exon 6 or replacing it with a GCN4 leucine zipper sequence depends on the COOH-terminal sequence.

Tropomyosins (TM) are a family of highly conserved coiled-coil actin binding proteins present in most eukaryotic cells. At least 15 different isoforms arise through the use of alternative promoters and alternative RNA splicing of the transcripts of a small number of genes (three to four in vertebrates; reviewed in Ref. 1). These isoforms are expressed in developmentally and tissue-specific patterns and differ in actin affinity.

A function common to all TMs is cooperative binding to F-actin (2). Tropomyosin molecules are aligned head-to-tail in the grooves of the helical actin filament (3, 4). The role of TM is best understood in striated muscle where it regulates Ca2+-dependent muscle contraction with Tn (reviewed in Refs. 5–7).

Structural studies have shown that Tn, found only in striated muscles, extends along at least the COOH-terminal third of the TM molecule (8–13). Troponin I, TnC, and the COOH terminus of TnT are positioned near Cys-190 of TM. The elongated NH2 terminus of TnT extends beyond the COOH terminus of one TM to the NH2-terminal 10–30 residues of the next TM along the actin filament (13, 14).

Troponin greatly increases the affinity of TM for actin in the presence of Ca2+, with a further increase upon removal of Ca2+ (15). Based on binding studies with TM and Tn peptides, Mak and Smillie (16) proposed that the α-TM-Tn interaction in the region of Cys-190 is weakened in the presence of Ca2+ (Ca2+-sensitive), whereas that at the COOH terminus and the overlap region is strong in the presence and absence of Ca2+ (Ca2+-independent). Substantial evidence has since supported this model.

The presence of tissue-specific alternatively expressed exons implies distinct functions for the regions of TM they encode. For example, of four exons in the α-TM gene encoding the COOH terminus (9a-9d), exon 9a, important for Tn interaction on the thin filament (17, 18), is uniquely expressed in striated muscles. Of the two sixth exons, only exon 6b is expressed in striated α- and β-TMs, although it is not restricted to striated isoforms. The exon 6b-encoded region of striated muscle TM (residues 189–214) is part of the putative Ca2+-sensitive Tn binding site.

To determine the requirements of the alternatively spliced exon 6 for TM function, we expressed a series of recombinant, unacetylated TM exon 6 deletion and substitution mutants and chimeras in Escherichia coli. Functional properties of the modified TMs were defined by actin affinity, TnT affinity, assembly with Tn on the thin filament, and regulation of the actomyosin MgATPase. We have shown that the region of TM encoded by exon 6 affects actin affinity but not TnT binding, assembly with Tn on the thin filament, or thin filament regulation. Portions of this work have been reported in a preliminary form (19).

MATERIALS AND METHODS

DNA Constructions and Protein Purification—General recombinant DNA techniques were performed as described in Sambrook et al. (20) or as recommended by the supplier.

Rat striated (pUC18/TM6b-9a) and rat smooth (pBR322/TM6b-9d) muscle α-TM cDNA clones were the gift of B. Nadal-Ginard (21).

Tropomyosin exon 6 (see Fig. 1A) variants were made from the rat smooth α-tropomyosin cDNA (pUC118/TM6b-9d) (21) by oligonucleotide-directed mutagenesis using a Bio-Rad Muta-Gene T7 in vitro mutagenesis kit (based on Kunkel et al. (22)). The nucleotides encoding 21 amino acid residues 191–211 were deleted from TM6b-9d resulting in TM6a-9d. These nucleotides were then replaced with residues 191–211 encoded by exon 6a, resulting in TM6a-9d. The nucleotides encoding amino acid

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† Recipient of a Predoctoral Fellowship from the American Heart Association, New Jersey Affiliate. Current address: Dept. of Biology, Florida State University, Tallahassee, FL 32306.

‡ To whom correspondence should be addressed: Dept. of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. Tel.: 732-235-4528; Fax: 732-235-4029; E-mail: hitchee@umdnj.edu.

1 The abbreviations used are: TM, tropomyosin; Tn, troponin; RU, response units; DTT, dithiothreitol; αH, Hill coefficient.

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\(\alpha\)-Tropomyosin's Exon 6 Affects Actin Affinity

Conformational analysis of tropomyosin exon 6 variants

| Exon variant | 20 °C | 28 °C |
|--------------|-------|-------|
| TM6b-9d      | 32.3  | 0.90  | 0.77 |
| TM6a-9d      | 24.0  | 0.78  | 0.34 |
| TM6a-9a      | 33.9  | 0.87  | 0.79 |
| TM6b-9a      | 34.6  | 0.87  | 0.71 |
| TM6a-9b      | 33.4  | 0.90  | 0.80 |

\(a\) The observed \(T_{m}\) is defined as the temperature at which the ellipticity at 222 nm, normalized to a scale of 0 (at 60 °C) to 1 (at 0 °C), is equal to 0.5.

\(b\) The fraction folded is defined as the ellipticity at 222 nm, normalized to a scale of 0 (at 60 °C) to 1 (at 0 °C where all TM6s are ~100% helical).

The TM/actin ratio determined using densitometry (arbitrary units) was normalized using the \(n\) reported by SigmaPlot. We have shown that saturation corresponds to a TM:actin molar ratio of 0.14, a stoichiometry of 1 TM:7 A.

**Real Time Kinetic (BIAcore) Analysis of TnT Binding to TM Exon 6 Variants**—A BIAcore (Pharmacia Biotech Inc.) biosensor was used to measure binding of TM in solution to immobilized TnT. Chicken muscle TnT was dialyzed against 10 mM Hepes, pH 7.0, 500 mM NaCl, 0.5 mM DTT at 4 °C overnight prior to immobilization on a sensor chip CM5 in the BIAcore system using an amine coupling kit (Pharmacia, Biosensor). Thirty \(\mu\)l of TM diluted to 0.33 mg/ml in 10 mM sodium borate buffer, pH 7.5, 300 mM NaCl were immobilized on the carboxylated matrix of a sensor chip activated by a 20-\(\mu\)l injection of a solution containing a 1:1 mixture of 0.2 M N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide and 0.05 M N-hydroxysuccinimide. Excess reactive groups were blocked with a 30-\(\mu\)l injection of ethanolamine hydrochloride.

Assays were carried out at 25 °C in a 5 \(\mu\)l/min flow of buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM MgCl\(_2\), 0.5 mM DTT) at 5 °C across a surface of immobilized TnT. The dissociation phase was initiated at 360–390 s by switching to continuous flow of buffer without TM, and finally, regeneration of the surface with 10 \(\mu\)l of 2.5 mM NaCl at 5 °C.

BIAevaluation software (Pharmacia, Biosensor, version 2.1) was used for data processing. Dissociation rate constants (\(k_d\)) were determined by fitting the dissociation data to the equation:

\[
R_t = R_{esp} + R_0 \frac{R_{esp} - R_0}{1 + e^{-kt}}
\]

which calculates the dissociation rate constants \((k_d)\) for the first phase of the bihapic curve; \(R_{esp}\) is the dissociation constant \((k_d)\) for the second phase of the bihapic curve; \(t_0\) is the start time for dissociation.

The part of the sensorgram usable for analysis is the most linear region, the region of \(R_t\) versus \(t\) (Eq. 1) which calculates the association rate constants \((k_a)\) and \(k_d\) for a heterogenous interaction, \(R_0\) is steady state, \(C\) is the molar concentration of TM; \(n\) is the steric interference factor. Generally, \(t_0\) was about 10–20 s and \(t\) was about 160–240 s after the start of elution (indicated by the region between the solid vertical lines in Figs. 5 and 6). Association rate constants \((k_a)\) were calculated from the calculated \(k_d\) values at different TM concentrations according to the following association type one model:

\[
R_0(1 + e^{-kt}) = L (1 + e^{-kt_d})
\]

which calculates the association rate constants \((k_a)\) and \(k_d\) for a heterogenous interaction, \(R_0\) is steady state, \(C\) is the molar concentration of TM; \(n\) is the steric interference factor. Generally, \(t_0\) was about 10–15 s and \(t\) was about 130–185 s after injection of TM (indicated by the region between the broken vertical lines in Figs. 5 and 6). Apparent binding constants \((K_{app})\) were calculated by dividing the average \(k_a\) (determined at different concentrations of each TM variant) by the average \(k_d\).

Applying the tests recommended by Schuck and Minton (36), our data are valid when evaluated using a two-site model.

**Actomyosin MgATPase Assay**—The actomyosin MgATPase was measured as a function of temperature (31, 32). The TM exon 6 variants showed multiple transitions with virtually the same degree of folding at 20 °C, with the exception of TM6a-9d which is only about 80% folded (Table I).

The actomyosin MgATPase was measured by cosedimentation at 25 °C in a Beckman model TL-100 centrifuge as described previously (18) with modifications (34). The bound and free TM were determined by quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue (34).

Apparent binding constants \((K_{app})\) and Hill coefficients \((n^2)\) were determined by using SigmaPlot (Jandel Scientific) to fit the data to the equation:

\[
v = \frac{n[M][T]}{K_{app} + [T]} = \frac{[M]^n}{K_{app}^n}
\]

(Eq. 1)

The observed \(T_{m}\) is defined as the temperature at which the ellipticity at 222 nm, normalized to a scale of 0 (at 60 °C) to 1 (at 0 °C), is equal to 0.5. The fraction folded is defined as the ellipticity at 222 nm, normalized to a scale of 0 (at 60 °C) to 1 (at 0 °C where all TM6s are ~100% helical).
FIG. 1. Tropomyosin exon 6 variants. The α-TM gene contains four regions encoded by alternative exons 1, 2, 6, and 9. A, all variants are identical to smooth rat α-TM (TM6b-9d) except for changes in the sixth exon. B, exon 9a was substituted for exon 9d in each of the exon 6 variants shown in A and renamed TM6a-9a, TM6b-9a, and TM6zip-9a. C, comparison of exon 6 sequences. Numbers indicate the amino acid position at the boundaries of the changes. Residues 168–192 of TM6a-9d were aligned with residues 189–213 of TM6b-9d, TM6a-9d, and TM6zip-9d. Identities are marked by an *; conservative substitutions are shown in lowercase letters.

RESULTS

Rationale—The alternatively spliced exon 6 of TM encodes residues 189–214 in striated TMs and is part of the putative Ca²⁺-sensitive Tn binding site. To define the requirements of the exon 6-encoded region for TM function, we made three exon 6 variants by deleting or replacing exon 6b in smooth TM (TM6b-9d, Fig. 1A). 1) Residues 191–212 of exon 6b were deleted (TM6a-9d). Only 21 of the 25 residues were deleted to maintain the heptapeptide repeat of hydrophobic residues important for the stability of the coiled coil. The deletion corresponds to one-half an actin binding period, based on there being seven quasi-equivalent periodic sites (40). 2) Residues 191–211 were replaced with the corresponding sequence from the alternative α-TM exon 6a (TM6a-9d). 3) The entire sixth exon, residues 189–213, was replaced with a non-TM coiled-coil sequence from the yeast transcriptional activator, GCN4 (TM6zip-9d) (23). Residue 212 in the original GCN4 sequence was changed from Gly to Ala to assure an uninterrupted coiled coil. These three exon 6 variants were expressed in E. coli to produce unacyetylated TMs with two different COOH-terminal exons: exon 9d, found in smooth and most non-muscle TMs (Fig. 1A) and exon 9a, unique to striated TM (Fig. 1B). All of the exon 6 variants contain the unique smooth α-TM exon 2a.

Actin Affinity of Tropomyosin Exon 6 Variants—The actin affinity of the TM exon 6 variants was measured by cosedimentation (Fig. 2, Table II). TM6b-9d (smooth muscle α-TM) bound to actin with high affinity, as previously reported (17, 18). Replacing 21 residues of exon 6b with 6a (TM6a-9d) resulted in a 2-fold increase in actin affinity, consistent with previous results (41). Replacement of exon 6b with a GCN4 leucine zipper sequence (TM6zip-9d) or deletion of half an actin binding period (TM6-9d) reduced the actin affinity so it could not be accurately measured. The effect of the leucine zipper replacement was surprising because the 284 residue length of the molecule and the coiled-coil α-helix were maintained. All of the exon 6 variants with an exon 9a-encoded COOH terminus bound weakly compared with TM6b-9d or TM6b-9d (Fig. 2, Table II), as does unacyetylated striated α-TM (17, 18). Surprisingly, deletion of exon 6 had little effect while replacement with the GCN4 leucine zipper sequence significantly reduced actin affinity. It is unclear how the effect of deleting 21 residues depends on exon 9.

Actin Affinity of Tropomyosin Exon 6 Variants in the Presence of Troponin—If the region encoded by exon 6 contributes to the Tn binding site on TM, one might expect the TM exon 6 variants to differ significantly in the effect of Tn on thin filament assembly. Troponin in the presence of Ca²⁺ caused a ~100-fold increase for all TM variants containing exon 9a (Fig. 3A), whereas it had only a small effect on the affinity of exon 9d-containing TMs, except TM6zip-9d and TM6a-9d which did not change (Fig. 3B). The 4-fold lower actin affinity of TM6zip-9a compared with TM6a-9a was a similar effect to that reported for striated α-TM with GCN4 sequence inserted in the second or third periods of the molecule (42). Removal of Ca²⁺ increased the affinity of all exon 9a- and 9d-encoded variants, independent of exon 6 identity (Fig. 3, A and B, and Table I).

To measure accurately the actin affinities in the absence of Ca²⁺, the NaCl concentration was increased from 150 to 300 mM to weaken actin affinity (Fig. 4). Together the results in Figs. 2–4 and Table II show that the relationship of the different exon 6 variants in terms of actin affinity remained constant with Tn in the presence and absence of Ca²⁺, as well as in the absence of Tn. Clearly, alterations in exon 6, or deletion of exon
**α-Tropomyosin’s Exon 6 Affects Actin Affinity**

6, primarily affect actin affinity, not Tn interaction on the thin filament. The results are consistent with previous reports showing the importance of exon 9 on Tn-enhanced TM affinity for actin (17, 18).

**Interaction of Tropomyosin Exon 6 and Exon 9 Variants with Troponin T**—From the actin binding experiments, we can only infer the requirements of exon 6 for Tn interaction because Tn binds to both actin and TM. Existing methods used to evaluate TM-Tn interaction are qualitative or require covalent modification of TM (16, 43, 44). Thus, we have developed a solid phase affinity assay employing biosensor technology to measure directly the binding of TM with TnT. In this assay, TnT was immobilized on a dextran matrix attached to a gold-coated sensor chip surface to detect binding of different TM variants in a flow chamber. The amount of TM which bound to the immobilized TnT was calculated from the surface plasmon resonance signal measured as a function of time (45). Using this technique, we have measured TnT binding of the exon 6 variants described here, as well as of a series of previously reported exon 9 variants (18).

Qualitative comparison of sensorgrams obtained from different TMs binding to the same surface can be quite informative. Figs. 5, A and B, shows the results when a 1.5 μM solution of each TM variant was allowed to bind to immobilized TnT. The data between the *vertical broken lines* were used to compare association rates and that between the *vertical solid lines* to compare dissociation rates.

Fig. 5A shows sensorgrams of the exon 6 variants. Comparing the association phases (data between the vertical broken lines) clearly shows that TM6Δ6-9a and TM6a-9a had the highest binding rates (steepest increase). Comparing the data between the vertical solid lines shows that TM6a-9a, TM6Δ6-9a, TM6Δ6-9d, TM6zip-9a, TM6zip-9d, TM6a-9d, TM6Δ6-9d, TM6zip-9a, and TM6zip-9d had similar (parallel) dissociation rates with significant residual signal at 480 s, reflecting measurable binding. In contrast, all the TM variants with exon 9d showed a very weak signal from 380–480 s, reflecting poor binding. In general, dissociation and rebinding (an inclination between the two vertical solid lines rather than a decline) was observed for the recombinant TMs which bound poorly to TnT.

Fig. 5B shows sensorgrams of a series of exon 9 variants, chimeras and deletion mutants in which the last nine residues of exons 9a and 9d were exchanged or deleted (18) (described here in the legend to Fig. 5B. All expressed exons 2b and 6b).
We previously reported that the last 9 residues encoded by exon 9d are important for actin binding whereas the striated specific exon 9a-encoded COOH terminus, especially the first 18 residues, are important for Tn-dependent thin filament assembly of unacetylated TM (18). Qualitative analysis of the sensorgrams in Fig. 5B shows that only TM9a and TM9a/9d, as well as acetylated striated α-TM isolated from muscle, bound to TnT in this assay. Replacing the first 18 residues of exon 9a with 9d (TM9d/9a), or deleting the last 9 residues (TM9a/), resulted in a major loss of TnT affinity, in contrast to deletion or replacement of exon 6 which had only a small effect. TM9d is particularly interesting because it appeared to have an extremely fast on-rate as well as an extremely fast off-rate, resulting in a biphasic curve as high surface occupancy is approached (14). Finally, two separate binding sites with different affinities may exist. However, we only detected binding to variants with exon 9a suggesting both sites must involve this domain.

Substitution of exon 6 with a non-TM coiled-coil sequence (TM6zip-9d) did not affect either the first or second binding phase. Deletion of the sixth exon (TMΔ6-9a) slightly reduced \( K_{\text{app}} \) but increased \( K_{\text{app}} \), 3-fold compared with the other exon 6 variants containing exon 9a. Clearly, the exon 6 sequence has little effect on TM affinity for TnT. The major determinant for TnT interaction is the COOH-terminal exon 9a, in particular the first 18 residues. The TM must be full-length, as deletion of the last 9 residues from exon 9a resulted in loss of binding. These results, summarized in Table III, are consistent with the actin binding studies (this work) (18).

In control experiments, binding of TM directly to a blank dextran matrix was 0–2 RU, demonstrating no measurable ionic interaction between the chip surface and TM. Therefore the fact that TMs which differ in sequence by as few as nine amino acids differ in the ability to bind TnT provides the best evidence that binding of TM variants to TnT is specific (compare TM9a with TM9a/ in Fig. 5 and Table III).

The acetylated muscle TM was not analyzed in detail because it polymerizes at 150 mM NaCl, complicating the interpretation and data analysis. By analyzing the sensorgram in Fig. 5B, it is estimated that acetylated TM binds TnT about 3-fold stronger than the unacetylated form (\( K_{\text{app}} \) values are equal). The affinity is similar to that reported by Lehrer and colleagues using a fluorescence assay (43, 44). This results suggests that acetylation of striated α-TM has only a small effect on TnT affinity, in contrast to the large effect (~100-fold) on actin affinity (35, 34, 47).

Calcium Regulation of the Actomyosin MgATPase by Tropomyosin Exon 6 Variants with Tropomin—The regulatory function of the TM exon 6 variants was evaluated by measuring their ability to confer calcium-dependent regulation of the actomyosin MgATPase with Tn. All TM variants that bound to Tn in the presence of Ca\(^{2+}\) inhibited the actomyosin ATPase in a Ca\(^{2+}\)-sensitive manner with similar effectiveness (Fig. 7). In contrast, TMΔ6-9d and TM6zip-9d, which did not bind to actin in the conditions of the ATPase assay, did not regulate. In the presence of Ca\(^{2+}\), the ATPase activity was the same with all variants. For simplicity, only TM6b-9d is shown in the presence of Ca\(^{2+}\). These results are consistent with previous reports.
from this laboratory that TMs that can bind to actin with Tn are similar in their ability to regulate the actomyosin ATPase (17, 18, 27, 48).

DISCUSSION

Analysis of a series of TMs that differ in the sequence encoded by exon 6 (residues 189–214) has shown that this region of TM influences actin affinity but not TnT binding or assembly and regulation with Tn on the thin filament. The two native TM sequences, encoded by exon 6a and 6b, both function normally with a striated or smooth/non-muscle COOH terminus, although there is about a 2-fold difference in actin affinity, as previously reported (41). However, the effect of deleting exon 6 or replacing it with a leucine zipper-encoding sequence depends on the COOH-terminal sequence; neither can bind to actin with an exon 9d-encoded COOH terminus in any condition tested. The results show that the sequence in the middle of TM can have a long range effect on the functional activity of the ends.

Of the 21 residues (residues 191–211) of exon 6b substituted with exon 6a sequence, 8 were identical, including four in a or d interface positions. Interestingly, whereas replacement of exon 6a for 6b in α-TM alters actin affinity (these results) (41), in β-TM it does not affect actin affinity (49), even though exon 6a and 6b are about 70 and 85% identical, respectively, in rat α-versus β-TM. Substitution of all 25 residues of exon 6 with the GCN4 leucine zipper sequence changed all but 5 residues, 2 of which are in interface positions. The observed loss of actin affinity of the 6zip variants in the absence of Tn was as great as previously reported for modifications at the NH2- or COOH terminus of TM (17, 18, 33, 47, 50). The results show that a native TM sequence in the exon 6-encoded region is critical for cooperative binding to actin. Without high resolution structural information about the TM structure, and its binding site on actin, further speculation is premature.

The most surprising result is that deletion of 21 residues of exon 6 abolished actin binding of TM with exon 9d but had little effect with a 9a-encoded COOH terminus. In previous work from this laboratory, a 21-residue deletion within exon 2b of a fusion striated α-TM (with exons 6b, 9a and an 80 residue fusion peptide on the NH2 terminus) resulted in loss of actin affinity (48). Because 21 residues correspond to one quarter of a turn of the coiled coil, we postulated that the failure of the 21-residue deletion to bind actin may be a consequence of altered geometry of the ends of adjacent molecules with respect to each other on the actin filament. However, the more deleterious effect of the 21-residue deletion in exon 6 in combination with exon 9d than exon 9a shown in this study implies that the structures of the ends are significant, but not simply in terms of the coiled-coil geometry. With our present understanding of TM structure we cannot explain how a 21-residue deletion has

FIG. 5. Tropomyosin variants binding to TnT. Twenty ml of TM (1.5 mM) at 25 °C in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 0.5 mM DTT was injected at 5 ml/min across a surface of immobilized TnT. The two vertical broken lines mark the region used for comparison of association and the two vertical solid lines mark the region used for comparison of dissociation. A, tropomyosin exon 6 variants which all have the smooth muscle specific exon 2a and B, tropomyosin exon 9 variants which all have exon 2b and exon 6b. In TM9a and 9d the COOH-terminal 27 amino acids are encoded by exons 9a and 9d, respectively. In TM9a/9d, the first 18 residues are encoded by exon 9a, the last 9 by exon 9d. In TM9d/9a, the first 18 residues are encoded by exon 9d, the last 9 by exon 9a. In TM9a/ and TM9d/ the last 9 amino acids have been deleted.

FIG. 6. Association and dissociation kinetics of A, TM6-9a; B, TM6a-9a; C, TM6zip-9a, D, TM9a; E, TM9a/9d with TnT covalently linked to CM-dextran through amino groups. Relative response (RU) was plotted as a function of time (s) for at four concentrations of the TM variants (1–12 mM). The two vertical broken lines mark the region of the binding curve used for calculation of association rate constants and the two vertical solid lines mark the region used for calculation of the dissociation rate constants.
The data from Fig. 6 were fit to the equation $R_t = R_{m}e^{-k_{d}t} + (R_{o} - R_{m})e^{-k_{d}t}$ which calculates the dissociation rate of two parallel reactions. The reported parameters were calculated as described under “Materials and Methods.”

| Tropomyosin | $k_{d1}$ | $k_{d2}$ | $K_{d1}$ | $K_{d2}$ | $K_{app1}$ | $K_{app2}$ |
|------------|----------|----------|----------|----------|------------|------------|
| TM6b-9d, TM6c-9d, TM6a-9d, TM6zip-9d | ND $^{a}$ | ND | $<10^5$ | ND | ND | $<10^5$ |
| TM6a-9a | $1.0 \pm 0.6 \times 10^{2}$ | $2.5 \pm 0.3 \times 10^{-2}$ | $4.0 \times 10^{5}$ | $2.7 \pm 1.7 \times 10^{2}$ | $6.1 \pm 1.2 \times 10^{-4}$ | $4.5 \times 10^{6}$ |
| TM6a-9a | $1.6 \pm 1.4 \times 10^{2}$ | $2.5 \pm 0.1 \times 10^{-2}$ | $6.3 \times 10^{5}$ | $2.6 \pm 1.3 \times 10^{2}$ | $2.0 \pm 0.2 \times 10^{-3}$ | $1.3 \times 10^{6}$ |
| TM6a-9d | $1.7 \pm 1.2 \times 10^{2}$ | $3.0 \pm 2 \times 10^{-2}$ | $6.0 \times 10^{5}$ | $3.9 \pm 2.4 \times 10^{2}$ | $2.3 \pm 0.7 \times 10^{-3}$ | $1.7 \times 10^{6}$ |
| TM6a | $4.5 \pm 3.1 \times 10^{3}$ | $3.2 \pm 0.2 \times 10^{-2}$ | $1.4 \times 10^{5}$ | $1.2 \pm 1.1 \times 10^{3}$ | $1.4 \pm 0.3 \times 10^{-3}$ | $8.5 \times 10^{5}$ |
| TM6a/9d | $6.1 \pm 3.9 \times 10^{3}$ | $3.5 \pm 0.6 \times 10^{-2}$ | $1.7 \times 10^{5}$ | $2.5 \pm 2.0 \times 10^{3}$ | $9.7 \pm 2.3 \times 10^{-4}$ | $2.6 \times 10^{6}$ |
| TM6a/9d, TM9d, TM9d/9a, TM9d/9d | ND | ND | $<10^5$ | ND | ND | $<10^5$ |

$^{a}$ ND, not determined.

FIG. 7. Effect of tropomyosin exon 6 variants on the actomyosin Mg$^{2+}$ ATPase in the presence of troponin without Ca$^{2+}$. Conditions: 2.4 μM actin, 0.6 μM myosin, 0–0.6 μM TM, 1 μM Tn in 40 mM NaCl, 5 mM imidazole, pH 7.0, 0.5 mM MgCl$_2$, 5 mM MgATP, 100 μM DTT, 0.2 mM EGTA for 15 min at 28 °C. Specific activity is expressed as μmol of P$_{m}$/mg of myosin/min. In the absence of the TM, the mean specific activity of the actin-Tn-myosin ATPase for all experiments pooled was $0.13 \pm 0.02$ μmol of P$_{m}$/mg of myosin/min (range = 0.09–0.16 μmol of P$_{m}$/mg of myosin/min; n = 35). Each curve contains normalized data pooled from three to four experiments. Data was normalized to the mean specific activity of the actin-Tn-myosin ATPase in the absence of TM for each experiment. Only TM6b-9d is shown with Tn and 0.2 mM DTT, 0.2 mM EGTA for 15 min at 28 °C. Specific activity is expressed as μmol of P$_{m}$/mg of myosin/min; n = 35). Each curve contains normalized data pooled from three to four experiments. Data was normalized to the mean specific activity of the actin-Tn-myosin ATPase in the absence of TM for each experiment. Only TM6b-9d is shown with Tn and 0.2 mM Ca$^{2+}$, since it is representative of all the TM exon 6 variants.

Of the leucine zipper replacement was similar to that reported for leucine zipper replacements at other sites in TM (42). The exception was TM6zip-9d where Tn had no effect in the presence of Ca$^{2+}$.

The effect of Tn on TM binding to actin correlates well with the binding of TnT to TM measured using biosensor technology where binding could be measured in all variants containing exon 9a, or the first 18 residues encoded by exon 9a in a full-length TM, independent of the exon 6-encoded sequence. The region of TM encoded by exon 6 (residues 189–213) has been implicated in Tn binding based on structural studies (11), binding studies with TM and Tn peptides (16), Tn, TnC, and TnT cross-linking to Cys-190 of TM (51, 52), and probes attached to Cys-190 responding to Tn and TnT binding to TM alone and on the actin filament (43, 44, 53, 54). However, the present results indicate that the interaction of TnT with TM is indifferent to the exon 6-encoded sequence ruling out this region of TM as a specific Ca$^{2+}$-sensitive Tn binding site. The interaction with Tn in this region must be much weaker than at the COOH terminus, and cross-linking and fluorescence studies may simply be detecting the close proximity between the exon 6-encoded region of TM and the Tn components.

Although exon 6 clearly is not required for interaction with Tn (unique to striated muscles), it may be important for interaction with caldesmon which is found in smooth and non-muscle cells. Some reports have suggested that a region of TM including exon 6 is important for caldesmon binding (55) and that the sequence of exon 6 influences the effect of caldesmon on the affinity of TM for actin (49). Also, further work will be necessary to understand the significance of isoform specific TM function in terms of its cooperative interaction with specific myosin isoforms on the actin filament. This is an essential TM function for which the structural requirements have not been investigated.

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