Independent Functions for the N- and C-Termini in the Overlap Region of Tropomyosin†

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ABSTRACT: Tropomyosin (TM) is a coiled-coil that binds head-to-tail along the helical actin filament. The ends of 284-residue tropomyosins are believed to overlap by about nine amino acids. The present study investigates the function of the N- and C-terminal overlap regions. Recombinant tropomyosins were produced in *Escherichia coli* in which nine amino acids were truncated from the N-terminal, C-terminal, or both ends of striated muscle α-tropomyosin (TM9a) and TM2 (TM9d), a nonmuscle α-tropomyosin expressed in many cells. The two isoforms are identical except for the C-terminal 27 amino acids encoded by exon 9a (striated) or exon 9d (TM2). Removal of either end greatly reduces the actin affinity of both tropomyosins in all conditions and the cooperativity with which myosin promotes tropomyosin binding to actin in the open state. N-Terminal truncations generally are more deleterious than C-terminal truncations. With TM9d, truncation of the N-terminus is as deleterious as both for myosin S1-induced binding. None of the TM9d variants binds well to actin with troponin ($\pm Ca^{2+}$). TM9a with the truncated N-terminus binds more weakly to actin with troponin ($-Ca^{2+}$) than when the C-terminus is removed but more strongly than when both ends are removed; the actin binding of all three forms is cooperative. The results show that the ends of TM9a, though important, are not required for cooperative function and suggest they have independent functions beyond formation of an overlap complex. The nonadditivity of the TM9d truncations suggests that the ends may primarily function as a complex in this isoform. A surprising result is that all variants bound with the same affinity, and noncooperatively, to actin saturated with myosin S1. Evidently, end-to-end interactions are not required for high-affinity binding to acto-myosin S1.

The actin-binding protein tropomyosin (TM)[1] is a two-chained coiled-coil expressed in nearly all eukaryotic cells that cooperatively binds to actin. Tropomyosin confers cooperative responses to other thin-filament functions including switching by myosin S1 to the open state and cooperative activation by Ca^{2+} binding to troponin (Tn) in striated muscle (*1*−*5*).

Tropomyosin molecules are aligned head-to-tail along the length of the actin filament (cf. refs *6* and *7*). Structural considerations led to the proposal that the N- and C-terminal ends of the 284-residue TM molecules found in muscle and long nonmuscle isoforms overlap each other by about nine amino acids. The overlap was proposed to explain the molecular length and the electron density and staining pattern of TM in crystals and paracrystals (*8*−*10*). The structure of the proposed overlap region is unresolved in the available X-ray structures (*11*−*13*), though the N-terminus does form a coiled-coil in a TM model peptide (*14*). An overlap of nine amino acids would reduce the effective length of TM from 284 to 275 residues, a length that is consistent with seven quasiequivalent actin binding sites averaging 39 $^1/_3$ amino acids (*9, 11, 15*). The ionic strength-sensitive viscosity observed for 284-residue TMs is primarily ascribed to the overlap of the ends because removal of the C-terminus by carboxypeptidase digestion and modifications of the N-terminus result in loss of viscosity (*16*−*18*).

The proposal of an overlap complex between TM molecules led to the idea that TM-dependent cooperative function was the result of TM−TM interaction through the ends. Modification or removal of the N- or C-termini from TM enzymatically or by mutagenesis is deleterious. The major effect seems to be on actin affinity rather than cooperative function, showing that at least part of the cooperativity has a different origin. Striated muscle TM lacking the C-terminal 9−11 amino acids has been the most extensively investigated. It has greatly reduced actin affinity though it cooperatively binds to actin with Tn (*19*−*22*). The Ca^{2+} dependence of activation of the regulated actomyosin S1-ATPase (with actin−TM−Tn) is cooperative, though the ATPase activity in the presence of Ca^{2+} is not as high as with a full-length TM (*23*, *24*). Removal of the C-terminal 11 amino acids also makes the binding of myosin S1-ADP to regulated actin less cooperative without Ca^{2+}, but cooperativity in the presence of Ca^{2+} was normal (*25*).

If the N- and C-terminal nine amino acids function *only* to form an overlap complex, then removal of either end should have comparable consequences. This is not so;

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[1] Abbreviations: TM, tropomyosin; Tn, troponin.

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N-terminal modifications have a greater effect than comparable C-terminal modifications (*26*). Removal of the C-terminal nine residues has different effects on striated muscle TM and a nonmuscle TM (TM2) (*22*). These TMs are identical except for the C-terminal 27 amino acids encoded by exon 9 (*27*). Striated muscle TM expresses exon 9a, a striated muscle-specific exon, whereas TM2, found in many nonmuscle cells, expresses exon 9d, found in smooth muscle and many nonmuscle TMs. Usually the effects of removing or modifying either the N- or C-terminus can be explained by the reduced affinity of the truncated TM for actin rather than direct cooperative interaction between TM molecules on actin (*19, 21, 28, 29*).

These and other findings have gradually led to the recognition that the ends of TM have independent roles in specifying isoform function. To understand the roles of the N- and C-terminal regions of TM, and the functional interaction between them, we have studied the effect of removing nine amino acids from the N-terminus, C-terminus, or both ends. We truncated two 284-residue rat α-TM isoforms that are identical except for the alternatively spliced C-terminal exon: striated TM and TM2, which we call TM9a and TM9d, respectively. In both cases, removal of the N-terminal nine residues is more deleterious than the C-terminal truncation on the cooperativity with which myosin S1 promotes binding of TM to actin in the open state. The single-ended truncations have a greater effect on the function of TM9d than TM9a. The effects are cumulative with TM9a, but removal of the N-terminus of TM9d is as severe as removal of both ends. Mutants of TM9a bind cooperatively to actin with Tn ($-Ca^{2+}$) despite complete removal of the overlap region. Surprisingly, the binding of all truncated forms of TM9a and TM9d to actin-S1 (no Tn) is noncooperative. Portions of this work have been published in a preliminary form (*30*).

MATERIALS AND METHODS

Construction of TM Truncation Mutants. Rat striated α-TM (TM9a) and TM2 (TM9d) cDNAs, constructed from clones that were the gift of Dr. B. Nadal-Ginard (*31*), were previously cloned into the pET11d expression vector (*22*). The C-terminal nine codons were truncated from TM9a and TM9d cDNAs and cloned into pET11d as previously described (*22*).

The nine N-terminal codons were removed from TM9a and TM9d by oligonucleotide-directed mutagenesis to construct $_{-9}$TM9a and $_{-9}$TM9d. The protocol of Stratagene ExSite PCR-based site-directed mutagenesis kit was followed, except *Pfu* DNA polymerase instead of *Taq* polymerase was used. Two synthetic oligonucleotides served as primers for PCR. One oligonucleotide, a 44-mer phosphorylated at the 5′ end (synthesized by Integrated DNA Technologies, Inc.), was complementary to the region of pET11d adjacent to the initiating ATG codon of the TM cDNA: 5′-GGTATATCTCCTTCTTAAAGTTAAACAA-AATTATTTCTAGAGGG-3′. The second oligonuclotide, 26 bases long (synthesized by Gibco−BRL), was complementary to the noncoding strand starting at the Met-10 codon and extending toward the 3′ end of the cDNA: 5′-ATGCT-GAAGCTCGACAAAGAGAACGC-3′. The resulting PCR product was shorter than the template by the 27 base pairs encoding TM residues 1−9, making Met-10 the new initiating codon. Following synthesis the product was ligated with T4 ligase and used for transformation of Stratagene Epicurian Coli XL1-Blue Supercompetent cells. The transformed cells were plated for colony isolation. Single colonies were reisolated and then used for DNA preparation. The mutation was confirmed by sequencing the entire cDNA on an ABI Perkin-Elmer-Cetus 277 Prism automatic DNA sequencer by the DNA Synthesis and Sequencing Facility, Robert Wood Johnson Medical School, UMDNJ. The mutagenesis retained the original TM sequence following deletion of the first nine amino acid residues. This mutant differed slightly from the N-terminally truncated TM9a previously studied (*26*), which was a deletion of residues 1−9 with Leu-11 changed to Val. When residue 11 was Val, the initial Met was removed following synthesis, resulting in a 10-residue deletion. In the present mutant the initial Met is retained.

Doubly truncated TM9a and TM9d were constructed by deleting nine codons for the N-terminal residues from pET11d/TM9a$_{-9}$ and pET11d/TM9d$_{-9}$ as described above.

Protein Expression and Purification. Recombinant tropomyosins were expressed in *E. coli* BL21(DE3)pLysS (TM9a$_{-9}$, $_{-9}$TM9a$_{-9}$, and $_{-9}$TM9d$_{-9}$) or BL21(DE3) ($_{-9}$TM9d) expression cells (*32*) and purified as described previously (*18*, *22*) except the $(NH_4)_2SO_4$ fractionation was 35−70% saturation instead of 35−60%.

Actin was isolated from White Leghorn chicken pectoral muscle acetone powder (*33*), polymerized by addition of KCl and $MgCl_2$ to 20 mM and 0.7 mM, respectively, and incubated at 37° C for 10 min before polymerization at room temperature. Myosin S1 was prepared by papain digestion of chicken pectoral myosin (*34*). Troponin was purified from chicken pectoral muscle (gift of Dr. J. Fagan, Rutgers University) according to the method of Potter (*35*), with modifications described in ref *29*.

The concentrations of actin, myosin S1, and Tn were spectrophotometrically determined with extinction coefficients at 280 (0.1%) of 1.1, 0.83, and 0.45, respectively. Concentrations of recombinant TM were determined by differential absorption spectra of tyrosine as previously described (*22, 36, 37*).

Actin Binding Assays. Tropomyosin binding to F-actin or actin saturated with S1 was measured by a cosedimentation assay as previously described (*18*) with modifications (*38*). The amount of bound and free TM in the pellets and supernatants, respectively, was quantified by densitometry of SDS−polyacrylamide gels (*39*) on a Molecular Dynamics model 300A computing densitometer. The apparent K_a of TM for F-actin was determined by fitting the experimental data to the equation with SigmaPlot (Jandel Scientific):

$$\upsilon = n[\mathrm{TM}]^{\alpha H} K_{app}{}^{\alpha H}/(1 + [\mathrm{TM}]^{\alpha H} K_{app}{}^{\alpha H}) \qquad (1)$$

where υ = fraction of maximal TM binding to actin, n = maximal TM bound, and $[TM] = [TM]_{free}$. The TM:actin ratio of the TMs that bound in the conditions of the experiment was normalized to 1.0 by dividing the TM:actin ratio obtained from densitometry by the TM:actin maximal ratio (n) from each experiment calculated with eq 1. The TM:actin ratio of TMs that did not bind measurably was normalized by use of the n value for TMs that bound. The

TM:actin density ratio at saturation was the same for all TMs that reached saturation in our experimental conditions. We have previously shown that the observed density ratio at saturation reflects stoichiometric binding of TM to actin (*26*). We have normalized the data because the intensity of the staining is somewhat variable from experiment to experiment.

Myosin S1-Induced Tropomyosin Binding to Actin. Actin (3 μM) and TM (1 or 3 μM, where indicated) in 30 mM NaCl, 0.5 mM $MgCl_2$, 1 mM DTT, and 10 mM imidazole, pH 7.0, were mixed with myosin S1 (0 to 3.6 or 4.2 μM). The mixture was incubated at room temperature for 0.5 h to ensure hydrolysis of residual ATP from F-actin and then centrifuged in a TLA-100 rotor for 25 min at 60 000 rpm, 20 °C, in a Beckman TL-100 ultracentrifuge. The pellets were washed with assay buffer and then solubilized in actin extraction buffer (5 mM imidazole, pH 7.0, 0.5 mM DTT, 0.1 mM $CaCl_2$, and 0.1 mM ATP) by sonication in an ultrasonic cleaner. The pellets were electrophoresed on SDS−12% PAGE gels (*39*). Proteins were visualized with Coomassie Blue. The composition of proteins sedimented in pellets was analyzed by densitometry. The results were plotted as the TM:actin and S1:actin ratio obtained from intensities of protein bands on the gel vs the initial S1:actin molar ratio.

The TM binding data were fit to eq 2, modified from eq 1, with SigmaPlot (Jandel Scientific):

$$\upsilon = \{n[\mathrm{X}]^{\alpha\mathrm{H}}K^{\alpha\mathrm{H}}/(1 + [\mathrm{X}]^{\alpha\mathrm{H}}K^{\alpha\mathrm{H}})\} + \mathrm{B} \qquad (2)$$

where υ = TM:actin ratio, [X] = S1:actin molar ratio, and B = TM:actin ratio without S1. The S1:actin ratio necessary for half-maximal saturation of actin with TM was equal to 1/K. The maximal TM:actin ratios for both C-terminally truncated TMs were the same, but this value was only ∼ 90% that of full-length TMs. N-Terminal and double truncations further reduced the maximal TM:actin ratio (being the same for $_{-9}$TM9d and $_{-9}$TM9d$_{-9}$ and lower for $_{-9}$TM9a$_{-9}$ than for $_{-9}$TM9a), indicative of incomplete saturation because of extremely weak actin affinity. Each TM−actin binding curve was normalized to 1.0 by dividing the observed TM:actin ratios by the maximal ratio ($n + B$) reported by SigmaPlot.

Circular Dichoism Measurements. CD measurements were made and analyzed with an Aviv model 62 DS spectropolarimeter as previously described (*40*).

RESULTS AND DISCUSSION

To understand the specific roles of the N- and C-terminal nine overlap residues of TM in thin filament function, we removed the first and/or last nine amino acids from striated muscle α-TM and from nonmuscle TM2, an alternatively spliced product of the α-TM gene (Table 1) (*27*). Both are 284 amino acids long and are identical except for the alternatively spliced exon 9 that encodes the C-terminal 27 residues, 258−284. Striated muscle α-TM (called TM9a) expresses exon 9a, and TM2 (called TM9d) expresses exon 9d. The N- or C-terminal removal of nine amino acids is indicated by a prefix or suffix, respectively.

All six mutant TMs were expressed at high levels in *E. coli* and were purified by standard methods (*18*, *22*). The N-terminal Met is not acetylated in *E. coli*. The lack of *N*-acetylation results in a large reduction in actin affinity and viscosity of TM9a (striated muscle α-TM) but not TMs expressing exon 9d (*18*, *22*, *38*). The secondary structure and thermal stability were evaluated by circular dichroism spectroscopy (*40*). All tropomyosin variants formed α-helical coiled coils and were ∼100% α-helical at 0 °C, but the T_ms of the variants were 1−3 °C lower than that of the comparable wild-type form. The effect of the mutations was local and did not alter the overall conformation and folding of the TMs (results not shown).

Table 1: Tropomyosin Variants

tropomyosin	mutation
TM9a (striated muscle)	full-length TM (284 residues) expressing striated-specific exon 9a
TM9d (TM2)	full-length TM (284 residues) expressing exon 9d
TM9a$_{-9}$	TM9a with last nine amino acids removed
TM9d$_{-9}$	TM9d with last nine amino acids removed
$_{-9}$TM9a	TM9a with first nine amino acids removed
$_{-9}$TM9d	TM9d with first nine amino acids removed
$_{-9}$TM9a$_{-9}$	TM9a with first and last nine amino acids removed
$_{-9}$TM9d$_{-9}$	TM9d with first and last nine amino acids removed

Myosin S1-Induced Binding of Tropomyosins to F-Actin. Removal of 7−10 amino acids from the N-terminus or 9−11 amino acids from the C-terminus of recombinant smooth or striated TM reduces actin affinity to the undetectable level (*22*, *26*, *41*). We have confirmed the results as part of the present study and have found, not surprisingly, that the TMs lacking both N- and C-terminal nine residues do not bind to actin (results not shown).

However, all forms will bind to actin in the presence of myosin S1. Myosin S1 increases the affinity of TM for actin (*29*, *42*, *43*). In the Geeves and Lehrer model, the binding of myosin heads to actin shifts the equilibrium of the actin-TM from the closed to the open, force-developing state in which both TM and myosin bind to actin with higher affinity (*5*, *44*, *45*). The sequences of the ends of TM are major factors in the cooperativity with which myosin S1 induces/promotes binding of TM to actin in the open state, as they are for actin affinity (*29*, *46*). Here we have investigated the requirement for ends and the presence of the overlap region for this TM function.

For each truncation mutant we have determined the number of myosin heads bound per seven-actin unit (the length of one TM molecule) required to switch the actin filament from the weak-TM-binding, closed state to the tight-TM-binding, open state. We have monitored the closed-to-open transition by a direct cosedimentation binding assay. A fluorescence change in TM to report the transition (*47*) was not used because the truncated TMs do not bind well to actin alone. We set the experimental conditions so TM binds weakly to actin without myosin S1 (10−20% saturation, depending on the TM affinity) but binds well when myosin S1 induces strong binding of TM to actin. In the conditions of these experiments, myosin S1 (no nucleotide added) bound stoichiometrically with very high affinity (Figures 1 and 2), saturating somewhat below the calculated 1:1 molar ratio, as is observed when myosin S1 binding to actin−TM is followed by light scattering (e.g., ref *47*).

The cooperativity with which myosin S1 promoted binding of TM9d to actin is greater than that for TM9a, requiring

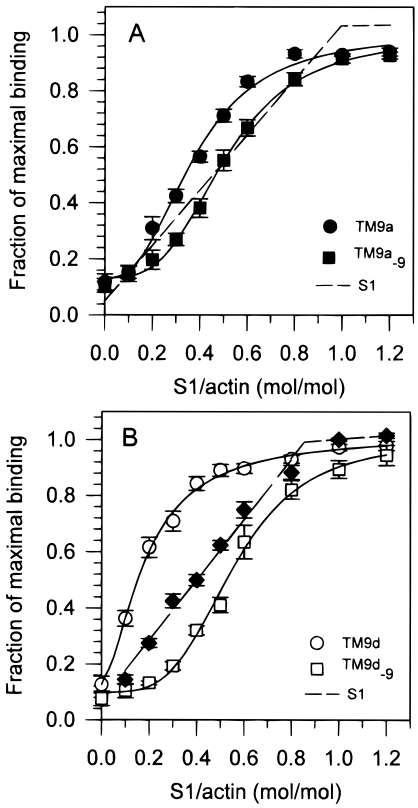

FIGURE 1: Myosin S1-induced binding of full length and C-terminally truncated TMs to actin. (A) TM9a (●) and TM9a$_{-9}$ (■); (B) TM9d (○) and TM9d$_{-9}$ (□). Binding of TM (1 μM) and S1 to actin (3 μM) was measured as a function of S1 concentration (0−3.6 μM) in 30 mM NaCl, 0.5 mM $MgCl_2$, 1 mM DTT, and 10 mM imidazole, pH 7.0, as described under Materials and Methods. The TM binding curves were fit to the data with the Hill equation. The myosin S1 binding to actin (- - -) was fit by a linear regression to the experimental points for S1:actin ratios 0−1.0 in panel A and 0−0.8 in panel B. The lines at saturation were drawn manually. For clarity the points (◆) are shown only in panel B. The points are the averages (±SEM) from four independent experiments.

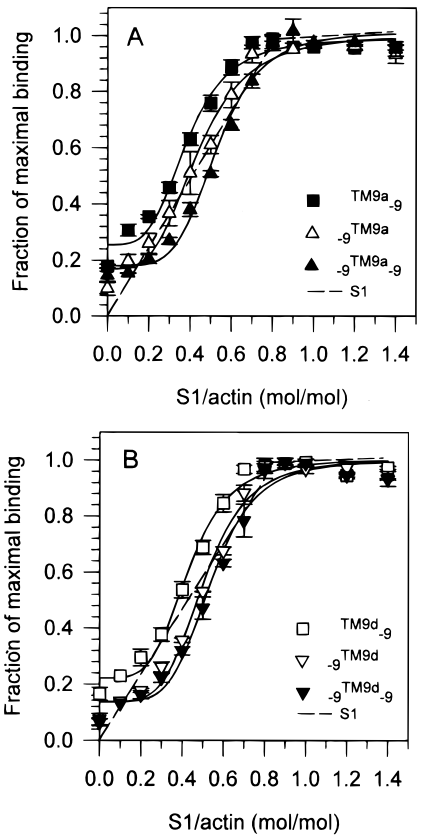

FIGURE 2: Myosin S1-induced binding of singly- and doubly truncated TMs to actin. (A) TM9a$_{-9}$ (■), $_{-9}$TM9a (△), and $_{-9}$TM9a$_{-9}$ (▲); (B) TM9d$_{-9}$ (□), $_{-9}$TM9d (▽), and $_{-9}$TM9d$_{-9}$ (▼). Conditions were as in Figure 1, except TMs were 3 μM and S1 concentration varied from 0 to 4.2 μM (see Materials and Methods). Myosin S1 binding to actin (- - -) is an average from three to four independent experiments, for clarity the points are not included. The myosin S1 line was fit by a linear regression to the experimental points for S1:actin ratios 0−0.8. The lines at saturation were drawn manually. The points are the averages (±SEM) from three or from four independent experiments.

1.2 versus 2.7 heads per seven actins (per one TM molecule) for half-maximal saturation (*29*; and Figure 1, Table 2). Removal of the overlap by C-terminal truncation greatly affects myosin S1-induced binding of TM in the open state, as it does actin affinity. Figure 1 shows that when the C-terminal nine amino acids were removed from TM9a (TM9a$_{-9}$) or TM9d (TM9d$_{-9}$), binding exceeded the basal level when two or more myosin heads bound per seven actin unit (per TM). Both half-saturated F-actin when the filament was about half-occupied with myosin S1 and saturated the actin filament only when it was fully occupied by myosin. Though full-length TM9d required lower myosin S1 occupancy for binding than TM9a, TM9d$_{-9}$ was slightly worse than TM9a$_{-9}$ in this function (Table 2). The results support other work showing that the C-terminal nine amino acids primarily define the isoform-specific functional differences between TM9a and TM9d (*22*, *29*).

Figure 2 shows the effect of the N-terminal and N- + C-terminal truncations on myosin S1-induced actin binding. In these experiments the TM concentration was 3 μM (versus 1 μM in Figure 1) to optimize TM binding to actin at the highest myosin S1 concentration. In some experiments the double truncation mutants only reached ~80% saturation of actin (~0.8 TM/7 actins versus 1 TM/7 actins, mol/mol). We did not include full-length TM9a and TM9d in Figure 2 because they bound too tightly to actin alone to measure the induction of binding by myosin S1.

Figure 2A (Table 2) shows the effect of single and double truncations on myosin S1-induced binding of TM9a to actin. Here the effects are cumulative: removal of nine amino acids from the N-terminus ($_{-9}$TM9a) was more deleterious than from the C-terminus (TM9a$_{-9}$). Removal of both ends had an even greater effect, requiring almost three myosin heads along the length of one $_{-9}$TM9a$_{-9}$ molecule for binding to exceed the basal level and almost four myosin S1 heads for half-maximal TM binding. The results correlated with the effect of the truncations on actin affinity with Tn (discussed later, Figure 4).

The effect of comparable truncations on TM9d was different from that on TM9a. While the cooperativity with which myosin S1 induced actin binding was greater with TM9d than TM9a, removal of either the N- or C-terminus from TM9d was more deleterious than with TM9a. Removal of the N-terminus alone from TM9d had the same effect as removal of *both* ends; the effect of the mutations was not cumulative (Figure 2B, Table 2). This is surprising since the C-terminal nine amino acids in TM9d that allow strong actin binding are present in $_{-9}$TM9d (*22*). Removal of both ends

Table 2: Binding of Tropomyosin Truncation Mutants to Actin in the Presence of Troponin or Myosin S1

type of TM	TM−actin binding constant, K_{app} (M^{-1})		S1/actin molar ratio[a]	number of S1 per 7 actin subunits[a]
	Tn, $+Ca^{2+}$	Tn, $-Ca^{2+}$		
TM9a	$(1.0 \pm 0.4) \times 10^7$	$>10^8$	0.38 ± 0.01[b]	2.7 ± 0.1[b]
$TM9a_{-9}$	$\ll 10^5$[c]	$(6.0 \pm 0.1) \times 10^6$[c]	0.38 ± 0.01	2.7 ± 0.1
			0.51 ± 0.02[b]	3.8 ± 0.3[b]
$_{-9}TM9a$	$\ll 10^5$	$(7.1 \pm 0.3) \times 10^5$	0.44 ± 0.04	3.1 ± 0.3
$_{-9}TM9a_{-9}$	$\ll 10^5$	$(3.3 \pm 0.2) \times 10^5$	0.53 ± 0.01	3.7 ± 0.1
TM9d	$(4.6 \pm 0.5) \times 10^6$[c]	$>10^8$	0.17 ± 0.02[b]	1.2 ± 0.2[b]
$TM9d_{-9}$	$\ll 10^5$[c]	$\ll 10^5$[c]	0.44 ± 0.02	3.1 ± 0.1
			0.56 ± 0.02[b]	3.9 ± 0.1[b]
$_{-9}TM9d$	$\ll 10^5$	$\sim 10^5$	0.51 ± 0.03	3.6 ± 0.1
$_{-9}TM9d_{-9}$	$\ll 10^5$	$\ll 10^5$	0.54 ± 0.01	3.8 ± 0.1

[a] The number of myosin heads per actin (or actin filament segment of 7 subunits) needed for half-maximal saturation (± S.E.M) corrected for TM binding in the absence of S1. The value is based on the initial S1/actin ratio, and is an overestimate of the cooperativity as S1 saturated actin at values below the calculated initial S1:actin ratio. This most likely because of inaccurate determination of protein concentrations. Data are averages from 3 to 4 experiments. Actin concentration was 3 μM or 1 μM (footnote *b*). [b] TM concentrations was 1 μM. [c] Data from ref *22*. The K_{app} values published are high by a factor of 2 because of a calculation error.

had the same effect on TM9a and TM9d (Figure 2, Table 2). Binding of $_{-9}TM9a_{-9}$ or $_{-9}TM9d_{-9}$ to actin over the basal level required ~40% occupancy by myosin S1 (almost three of the seven actins along the length of one TM molecule), and >50% saturation (almost four of the seven actins along the length of one TM) for half-maximal binding.

The results show that elimination of the inter-TM overlap by removal of the nine residues at either end of TM, or both ends, does not eliminate myosin S1-induced binding of TM to actin, though it greatly reduces actin affinity. When either end is removed from TM, regardless of the C-terminal sequence, more than one myosin S1 per TM molecule must bind to actin for TM binding to exceed the basal level. The actin must be half-saturated or more with myosin S1 for half-maximal TM binding. Therefore, if myosin S1 binds non-cooperatively and randomly, the information about myosin S1 binding is not carried for long distances through the actin filament, beyond the length of a single TM molecule. By this definition, the cooperativity of the myosin effect is lost. The sigmoid shape of the binding curve may have more than one explanation, not relating to cooperativity in the traditional meaning. Random binding of myosin S1 to F-actin does not imply a uniform distribution at low occupancy. In conditions where a binding stoichiometry of more than one myosin S1 per seven actins is required for TM to bind, a lag phase is anticipated before there is measurable TM binding. The sigmoidicity may also reflect that the actin affinity of the truncated TMs depends on, but is not directly proportional to, myosin S1 occupancy of multiple actin subunits along the length of one TM molecule. Alternatively, myosin S1 at subsaturating concentrations may bind nonrandomly along the actin filament, although stoichiometrically in the absence of ATP. A clustering of myosin S1 may result from local, cooperative changes in the actin filament caused by myosin binding. Tropomyosin would then preferentially bind to regions on the filament with the highest myosin S1 occupancy. The mode of myosin S1 binding to F-actin remains to be more fully described. At the higher levels of myosin S1 and TM saturation there may also be steric considerations that negatively affect the ability of TM to saturate the actin filament.

Myosin binding to F-actin changes the filament in some way to allow high-affinity TM binding in the open state, even in the absence of the TM ends. The ends of TM9a have

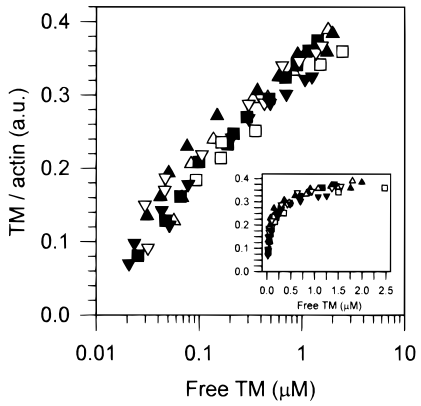

FIGURE 3: Binding of N-terminally and doubly truncated TMs to acto-S1. Tropomyosin at concentrations from 0 to 3 μM was cosedimented with 2.5 μM actin and 2.5 μM S1 in 150 mM NaCl, 10 mM imidazole, pH 7.5, 2 mM $MgCl_2$, and 0.5 mM DTT as described under Materials and Methods. The ionic conditions here are more favorable for TM binding to actin than those in Figures 1 and 2. Both full-length TMs bind too tightly to acto-myosin S1 to measure. The TM:actin ratio from densitometry were not normalized. The data shown in the linear plot (inset) demonstrate that all TMs reached saturation. The symbols are the same as in Figures 1 and 2. The data are from one representative experiment.

independent functions in TM binding to actin since the effect of removal of the ends is cumulative; this would not be expected if they solely functioned as a complex. In contrast, removal of either end from TM9d greatly affects the myosin occupancy of actin required to induce TM binding. The effects are not additive, implying functional dependence of one end of TM9d on the other, consistent with a requirement of direct interaction between the ends. The viscosity of smooth TM is greater than that of skeletal TM, evidence for stronger head-to-tail interaction of TM isoforms expressing exon 9d (*48*−*50*)

Affinity of Tropomyosin N- and C-Terminal Truncation Mutants for Actin−*Myosin S1*. Myosin S1 increases the actin affinity of TMs we have studied more than 500−1000-fold, to a level that is too tight to measure (*29*). The actin affinity of the truncated TMs, however, is sufficiently weak that we were able to measure TM binding to F-actin saturated with myosin S1 (Figure 3). The affinity of all truncated forms was $\sim 10^7$ M^{-1} and the binding was not cooperative. We were surprised by this result because it means that myosin alters the actin filament such that an individual TM molecule can

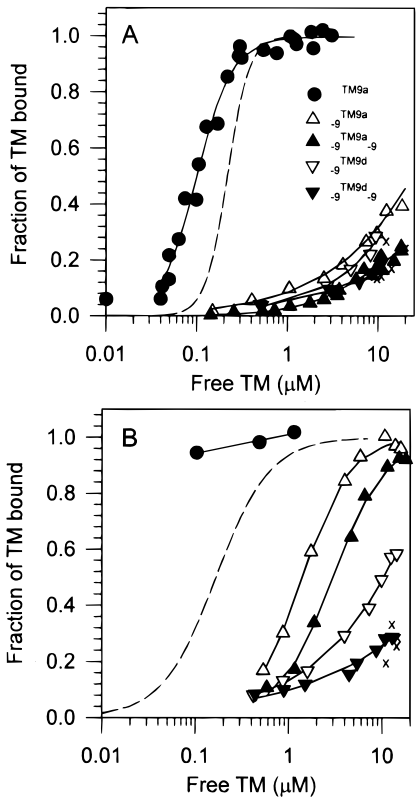

FIGURE 4: Binding of N-terminally and doubly truncated tropomyosin mutants to actin in the presence of troponin. Tropomyosin at concentrations from 0 to 18 μM (0 to 3 μM for wild-type TM9a) was cosedimented with 5 mM actin in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM $CaCl_2$ (A) or 0.2 mM EGTA (B). Troponin concentration was at 1.2-fold molar excess over TM. Although Tn was in excess, the affinity of the truncated TMs for Tn is probably too weak to allow TM−Tn binding in the conditions of these experiments, even at the highest concentrations tested (22 μM Tn). The curves were fit to the data with the Hill equation. Symbols: TM9a (●), $_{-9}$TM9a (△), $_{-9}$TM9a$_{-9}$ (▲), $_{-9}$TM9d (▽), and $_{-9}$TM9d$_{-9}$ (▼), and truncated TMs without Tn, a measure of trapping (×). Dashed lines represent binding of TM9d (A) and TM9a$_{-9}$ (B) from the binding parameters published in ref (*22*). TM9a$_{-9}$ binds very weakly in the presence of Ca^{2+} (*22*), as do the other truncated forms of TM9a in panel A. In the absence of Ca^{2+}, TM9d, like TM9a, binds too tightly to measure (*22*).

bind tightly. End-to-end interactions between TM molecules are not required for high-affinity binding (K_{app}) to acto-S1 as they are for binding to actin alone or in the presence of Tn (*51, 52*). Although the ends of TM are needed for very high affinity binding to acto-S1, it is not known if the binding of full-length TMs to acto-S1 is cooperative because the affinity is too high to measure. Interestingly, the differential effect of removing the N- and C-terminal ends was not apparent as it was with myosin S1-induced binding or with troponin-induced binding (see below).

Actin Affinity of N- and C-Terminal Truncation Tropomyosin Mutants with Troponin. Troponin increases the affinity of TM for actin through its direct interaction with both TM and actin. We have shown that Tn increases actin affinity and binds to TM9a but has little effect on TM9d (*22, 50, 53*). In the presence of Ca^{2+}, the affinity of all mutant TMs was weak, at or slightly above the level of trapping and nonspecific binding (Figure 4A; *22*). In the absence of Ca^{2+}, Tn increased the affinity of all three TM9a forms (Figure 4B; *22, 26*). The actin affinity of $_{-9}$TM9a was lower than that of TM9a$_{-9}$, and both were much lower than full-length TM9a (*22, 50*). As with the myosin S1-induced binding, $_{-9}$TM9a$_{-9}$ bound less well than either $_{-9}$TM9a or TM9a$_{-9}$; the effects were cumulative. Although binding of $_{-9}$TM9a and $_{-9}$TM9a$_{-9}$ was extremely weak, like TM9a$_{-9}$ it saturated actin and binding was cooperative [Hill coefficients: $_{-9}$TM9a, 1.7 ± 0.1; TM9a$_{-9}$, 1.5 ± 0.2 (from ref *22*); $_{-9}$TM9a$_{-9}$, 1.5 ± 0.1].

In contrast, the results for the Tn-induced actin binding of TM9d do not parallel the myosin S1-induced binding. Tn had a small effect only on $_{-9}$TM9d; binding of TM9d$_{-9}$ and $_{-9}$TM9d$_{-9}$ was at the level of trapping even with Tn in the absence of Ca^{2+}, consistent with the requirement for the C-terminal nine residues encoded by exon 9d for strong actin binding (Figure 4B, *22*). We surmise that binding of the TM9d truncated forms is unmeasurable because cooperativity between the ends is lost. The ability of Tn to promote actin binding of $_{-9}$TM9a$_{-9}$ but not $_{-9}$TM9d$_{-9}$ reflects the importance of all of exon 9a, the exon expressed specifically in striated muscle that is specialized for interaction with Tn (*22, 50, 53*). For full function of Tn, however, both the N- and C-terminal nine residues must be present, even though the N-terminal sequence encoded by exon 1a is not isoform-specific.

CONCLUSIONS

The results from the present study comparing the consequences of removal of the ends from two TM isoforms infer distinct aspects to the mechanisms of cooperative thin-filament function. We propose that the highly cooperative activation of actin-TM9d (either TM2 or smooth TM), (*29, 46, 54*) from the closed to the open state by myosin S1 depends on end-to-end interaction, in agreement with the proposal of Lehrer et al. (*46*). Supportive evidence comes from our observation that deletion of either end of TM9d has a large effect on myosin S1-induced binding of TM to actin; N-terminal truncation is as deleterious as removal of both ends.

On the other hand, although striated TM9a requires higher myosin S1 occupancy of actin to switch the thin filament from the closed to the open state than TMs expressing exon 9d (smooth TM, TM2) (*29, 46*), we show here that the effects of N- and C-terminal deletions are cumulative. The results imply that, unlike TM9d, end-to-end interaction may be weak without Tn. Troponin T, in particular the N-terminal domain that binds to the overlap region of TM, facilitates the cooperative activation of the thin filament to the open state by myosin S1 (*55*). We suggest that TnT compensates for the weaker end-to-end interaction of TM9a by promoting direct or indirect intermolecular TM communciation on the actin filament. The binding site of TnT on TM includes the exon 9a-encoded sequence of TM (cf. refs *20, 53,* and *56*−*58*) and extends to the N-terminus of the next molecule (*59*−*63*). A fuller understanding of the mechanisms of TM and TM-Tn dependent cooperative thin-filament activation will require knowledge of the structures of the ends of TM and of the TnT complex with the TM9a ends.

The most surprising outcome of the present study is our finding that truncated TMs exhibit noncooperative but high-affinity binding to actin saturated with myosin S1. While

this rigor state would not be found in a living cell, it does emphasize that myosin binding to actin must change the structure of the actin filament, or the actin monomers within the filament, to allow TM to bind. Since the truncated TMs bind noncooperatively and require high myosin S1 occupancy of actin to bind, the effect must be local and is likely to affect the actual TM binding site on the thin filament. We are reluctant to speculate further since at the present time there is little knowledge about changes in actin that result from TM or myosin binding, and we lack atomic-resolution structures of the thin filament.

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