

Integral Repeats and a Continuous Coiled Coil Are Required for Binding of Striated Muscle Tropomyosin to the Regulated Actin Filament*

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Tropomyosin is a coiled-coil protein that binds along the length of filamentous actin and contains sequence repeats that correspond to actin monomers in the filament. Analysis of striated muscle α -tropomyosin mutants in which internal sequence has been deleted or replaced with non-tropomyosin sequence showed that the following parameters are important for high affinity, cooperative binding of tropomyosin-troponin to actin. 1) Tropomyosin must be a coiled coil along its entire length. 2) An integral number of repeats corresponding to the actin monomers along its length is more important than the total number. 3) In comparison, the actin affinity is relatively insensitive to changes in the sequence of the internal regions of tropomyosin. The results suggest that the internal sequence repeats function as weakly interacting spacers to allow proper alignment of the ends on the regulated actin filament.

Periodic patterns of amino acids are a common feature of fibrous and structural proteins including collagen, tropomyosin (TM),¹ spectrin, and nebulin. Certain periodicities are crucial for formation of the basic molecular structure of the protein, as the Gly-X-Y repeat is for the collagen triple helix (1) and the heptapeptide repeat of hydrophobic amino acids is for the coiled coil (2, 3). Some fibrous proteins contain additional repeats that have been postulated to be related to assembly of higher order structures, such as those in TM for actin binding (4–6) and those in myosin for filament assembly (7). The present study addresses 1) the importance of a 7-fold repeat and 2) the sequence requirements for binding of striated muscle α -TM to the regulated actin filament (actin with troponin (Tn)).

Tropomyosins form a family of highly-conserved actin binding proteins found in virtually all eucaryotic muscle and non-muscle cells (8, 9). Tropomyosin is a two-chained, parallel coiled coil along its entire length except for the ends whose structure is unknown (10, 11). It is localized in the long pitch grooves of the helical actin filament (12, 13). Functions common to TMs are to bind cooperatively to F-actin, to stabilize and stiffen the actin filament, and to allow cooperative activation of the actin filament by myosin (14–17). In striated muscle, the TM-Tn complex regulates actin-myosin interaction in a Ca^{2+} -

dependent fashion (18, 19).

The lengths of TM isoforms correspond to an integral number of actin monomers in the filament: seven in 284-residue TMs found in muscle and certain nonmuscle isoforms, six in 247-residue nonmuscle isoforms, and five in yeast TMs (8, 9). This relationship implies the presence of periodic binding sites that correspond to the number of actin monomers spanned by a single TM molecule on the actin filament. McLachlan and Stewart (5) and Phillips (6) identified a poorly-conserved 7-fold periodic repeat of amino acids in striated α -TM that is sufficiently regular to correspond to actin binding sites. To test their hypothesis, we previously made a series of nested deletions in the chicken striated α -TM cDNA in the region encoding the second actin binding site (residues 47–88). The deletions corresponded to one-half, two-thirds, and one actin binding site, based on there being seven sites (repeats) per TM molecule (20). Analysis of these mutants showed that an integral 7-fold periodicity is important for binding of TM-Tn to actin. Here we address, first, whether the repeats function primarily as quasi-equivalent actin binding sites or as weakly interacting spacers to ensure the proper alignment of the ends (known to be important for actin binding) relative to each other and to actin monomers on the regulated actin filament. Second, we determined the structural requirements of the repeats for cooperative binding of TM to the regulated thin filament.

EXPERIMENTAL PROCEDURES

Construction of Tropomyosin Mutants—Deletion and replacement mutations were made of a chicken striated α -TM cDNA cloned in M13mp18 (Ref. 21, a gift of the late A. R. MacLeod) using oligonucleotide-directed mutagenesis (22) following a protocol modified from a Bio-Rad Muta-Gene *in vitro* mutagenesis kit as described previously (20). Following plaque purification, the sequence of one strand of the entire cDNA sequence was determined (23). All cDNAs were cloned in pET11d at the *Nco*I and *Bam*HI sites and transformed into *Escherichia coli* strains BL21(DE3) or BL21(DE3)pLysS for expression (24).

The nucleotides encoding amino acid residues 89–123 (dAC3–35), 86–127 (dAC3–42), 47–123 (dAC23) and 47–60 (dAC2–14) were deleted using the following oligonucleotides (antisense sequence, the slash indicates the site of the deletion): dAC3–35, 5'-ACCTTCATTCCTCTTTC/CAGGGAAGTACTTC-3'; dAC3–42, 5'-GCTCTATTTTCAATGACCTT/ACTTCACTCTCAGC-3'; dAC23, 5'-GACCTTCACTTCTCTTTC/CAGAGCCACCAGC-3'; dAC2–14, 5'-ATCTTTAAGGGACTCGGA/CAGAGCCACCAG-3'. The dAC2 mutant was described in Ref. 20. The replacement mutants were made using the following oligonucleotides (antisense sequence, the slash indicates the site of the deletion): 2zip, 5'-ATCTTTAAGGGACTCGGAAGATGGTAGTTTTTAGAAAGAA-GTTCTTCACTTTGTCTCACAGAGCCACCAG-3'; 3zip, 5'-GCCA-AGCGTCTCTGAAGATGGTAGTTTTAGAAAGAAAGTTCTTCA-CTTTATCTTCCAGGGAAGCTAC-3'; 2rc, 5'-ATCTTTAAGGGACTCGGAGTAACCGTCACCTTTACGACCATCGCCTTCACGACCGTC-GCCAGAGCCACCAG-3'; 3rc, 5'-GCCAAGCGTCTCTGGTAACCGTTCACCTTACGACCATCGCCTTCACGACCGTCGCCAGGGAA-GCTAC-3'.

Protein Purification—Recombinant TM was purified from *E. coli*

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¹ The abbreviations used are: TM, tropomyosin; Tn, troponin.

TABLE I
Binding parameters of tropomyosin-troponin interaction with filamentous actin

The data in the binding isotherms in Figs. 1B and 2B were analyzed using two methods to obtain values for the affinity and cooperativity. The Hill equation ($v = [nTM]^{\alpha_H} K_{app}^{\alpha_H} / 1 + [TM]^{\alpha_H} K_{app}^{\alpha_H}$) is independent of a specific molecular model and reports the apparent binding constant (K_{app}) and the Hill coefficient (α_H), a measure of cooperativity. The data were analyzed using a linear lattice model (38) to determine the affinity of tropomyosin for an isolated single site on the actin filament (K_o) and a cooperativity parameter (y). In this model, the cooperativity results from the binding of a tropomyosin to site on the filament that is contiguous to an occupied site. K_{app} is comparable to yK_o . A K_{app} of $<0.1 \times 10^6 M^{-1}$ is below the detectable level. Both analyses were carried out using commercially available programs (SigmaPlot and MATLAB), and the parameter values and standard deviations are those reported for the data in Figs. 1B and 2B. The linear lattice analysis was done by Dr. L. S. Tobacman, University of Iowa.

TM	K_{app} $10^{-6} \times M^{-1}$	Hill coefficient	K_o $10^{-4} \times M^{-1}$	y	yK_o $10^{-6} M^{-1}$
Wild type	9.6 ± 0.5	6.0 ± 1.6	4.2 ± 1.0	227 ± 57	9.5
dAC2	1.4 ± 0.1	2.5 ± 0.4	3.1 ± 0.3	40 ± 4	1.2
dAC2-14	<0.1				
dAC3-35	0.53 ± 0.06	2.7 ± 0.7	0.94 ± 0.2	52 ± 11	0.49
dAC3-42	0.48 ± 0.04	2.5 ± 0.6	1.1 ± 0.2	39 ± 6	0.44
dAC23	4.4 ± 0.3	5.3 ± 1.5	2.8 ± 0.7	151 ± 36	4.2
2zip	1.7 ± 0.2	3.4 ± 1.2	1.8 ± 0.6	92 ± 30	1.6
3zip	1.3 ± 0.1	2.7 ± 0.5	2.1 ± 0.2	55 ± 7	1.2
2rc	<0.1				

extracts as described previously (20). Briefly, TM was purified from the heat-stable fraction by chromatography on DE52 cellulose (Whatman) and hydroxylapatite (Bio-Rad). Conformation and stability analysis using circular dichroism (222 nm) showed that the observed T_m and fraction folded at 20 °C were as follows: wild type, 42.6°, 94%; dAC2, 43.4°, 88%; dAC3-35, 37.1°, 89%; dAC3-42, 36.4°, 87%; dAC23, 37.0°, 89%; 2zip, 43.8°, 85%; 3zip, 43.9°, 93%; 2rc, 37.2°, 79% (25).²

Chicken pectoral actin and Tn were purified as described previously (26, 27).

Actin Binding Measurements—The actin affinity of TM-Tn was carried out by cosedimentation in a Beckman model TL-100 centrifuge in a TLA 100 rotor at 60,000 rpm for 25 min at 20 °C in 200 mM NaCl; 2 mM MgCl₂; 0.2 mM CaCl₂; 20 mM imidazole; 0.5 mM dithiothreitol; 2.5 μM chicken pectoral actin; chicken pectoral Tn and TM in a 1.2:1 molar ratio, 0 to 2–15 μM, depending on the TM. The conditions differ from previous analyses (28, 29) in the NaCl concentration (200 versus 150 mM) and the constant Tn/TM ratio. These modified conditions allowed all the TMs to be compared in the same experiment.

The observed K_{app} values differed depending on the ionic strength and other experimental conditions employed in the course of the research. However, the order of actin affinities of the TM variants remained constant over time and at the two ionic strengths with the exception that dAC3-35 was close to that of dAC2 at 150 mM, whereas in 200 mM NaCl, it was similar to dAC3-42. TMs from different preparations had indistinguishable affinities.

The bound and free TM were determined by quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue (29, 30). Bound TM was expressed as the fraction of maximal binding, based on the TM/actin ratio in the pellets. Free TM was determined by the analysis of TM in the supernatant, using chicken pectoral α-TM as a standard. The parameters reported from curve fitting are in Table I.

Protein concentrations were determined using a microbiuret assay with bovine serum albumin as a standard (31) or by determining the specific tyrosine absorbance (32). Extinction coefficients (1% at 280) were used to calculate the concentration of actin (11.0) and Tn (4.5).

RESULTS AND DISCUSSION

Actin Binding Site Deletion Mutants—Deletion of site 2 (dAC2, 42 amino acids, residues 47–88) reduced the actin affinity of TM-Tn two- to seven-fold, depending on the ionic conditions (Fig. 1B; Table I; Ref. 20). To learn if the different TM repeats contribute in an equivalent way to the overall actin affinity, we deleted the third actin binding site, away from the ends and the Tn binding site (33, 34). The deletions were 35 amino acids (dAC3-35; residues 89–123) and 42 amino acids (dAC3-42; residues 86–127), multiples of seven to retain the heptapeptide repeat important for coiled coil formation (2, 3). Consequently, the deletions do not correspond perfectly to the 39½-residue repeat observed by McLachlan and Stewart (5).

Considering the helical (azimuthal) position on the α-helix as well as the linear (supercoil) position of each amino acid, Phillips (6) postulated that the repeats were 42 (e.g. site 2) or 35 residues (e.g. site 3), with an average of 39½ amino acids.

The mutants were expressed in *E. coli* to produce TMs unacetylated at the N-terminal Met (35). N-Acetylation is required for striated α-TM to bind tightly to actin, but in the presence of Tn, unacetylated TM binds well and regulates the actomyosin ATPase (28, 29, 35, 36). We measured the actin affinity of TM in a TM-Tn complex with saturating Ca²⁺. The data were analyzed using the Hill equation as well as a linear lattice model (37, 38). The K_{app} values of both site 3 mutants for regulated actin were indistinguishable, 2.7-fold weaker than dAC2, and almost 20-fold weaker than wild type (Fig. 1B; Table I). The mutant TMs were reduced in both affinity of TM-Tn for an isolated site on actin (K_o) and cooperativity (α_H or y). The main effect of the dAC2 deletion was reduced cooperativity. The dAC3 mutants had lower actin affinity than dAC2 without further reduction in cooperativity, implying that site 3, a highly conserved region of TM, is more critical for actin binding than site 2.

If each repeat (site) contributes individually to the overall actin affinity, then deletion of two sites should reduce the actin affinity more than deletion of one site. In dAC23, 77 amino acids corresponding to sites 2 and 3 were deleted (Fig. 1A, residues 47–123), close to the length of two actin binding sites according to the McLachlan and Stewart model (78½ residues; Ref. 5), and the same as in the Phillips proposal (6). Surprisingly, the K_{app} of dAC23 TM-Tn for actin was reduced only 2-fold compared with wild-type TM, and the cooperativity was essentially unchanged. The K_o of dAC23 was close to that of dAC2, considerably higher than either dAC3 mutant, suggesting that the number of repeats is not a major determinant of actin affinity.

The TM mutants of different lengths typically saturated the actin filament at the same TM/actin mass ratio. This implies that at saturation the TM-Tn complexes are aligned head-to-tail along the actin filament, independent of the length of the TM. Considered in terms of mass (versus the molecular weight of the TM), the K_{app} of dAC23 is 62% of wild type (versus 46% when calculated in terms of molecular weight), while the K_o is 93% of wild type (versus 67%). The Tn/TM ratio at saturation was also proportional to the length of the TM, consistent with one Tn binding site/TM on the thin filament.

The high affinity and cooperativity of dAC23 relative to the dAC2 or dAC3 mutants suggests that individual repeats (sites)

² N. J. Greenfield, unpublished results.

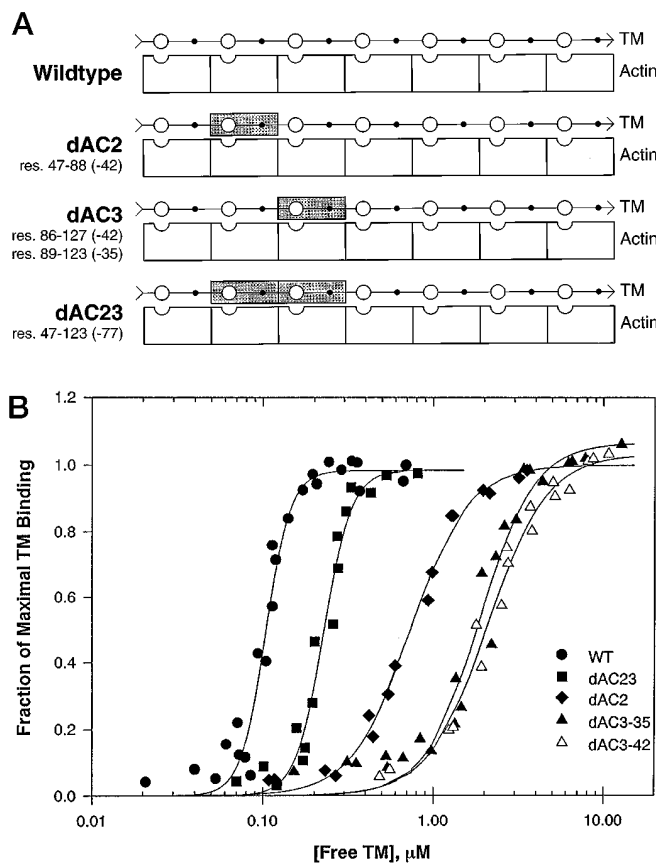


FIG. 1. Actin binding site deletion mutants. *A*, design. Integral actin binding repeats were deleted resulting in TMs that span five or six actin monomers on the filament, versus seven in wild-type TM. The drawing shows one TM and the actin monomers along its length in one strand of the helical actin filament, each actin with a hypothetical TM binding site. The N terminus of TM is at the left. The large and small circles relate to the α - and β -actin binding sites postulated by McLachlan and Stewart (5). Troponin is not illustrated. Both dAC2 and dAC3, deletion of site 2 or 3 respectively, give rise to TMs that should span six instead of seven actin monomers. In dAC23, both sites 2 and 3 were deleted, and the resulting TM should span five actin monomers. In the filament, the TM molecules would be aligned head-to-tail along the length of the filament. *B*, actin binding of tropomyosin-troponin, with Ca^{2+} . Recombinant, unacetylated TM was cosedimented with actin at 20 °C in 200 mM NaCl; 2 mM MgCl_2 ; 0.2 mM CaCl_2 ; 20 mM imidazole, pH 7.0; 0.5 mM dithiothreitol; 2.5 μM chicken pectoral actin; chicken pectoral Tn and TM in a 1.2:1 molar ratio, 0 to 2–15 μM , depending on the TM, as described under “Experimental Procedures.” Bound TM is expressed as the fraction of maximal binding, based on the TM/actin ratio in the pellets. The parameters reported from curve fitting are in Table I. Each curve is calculated from the data from two independent experiments, except for WT, which is from three experiments.

contribute little to the affinity of TM-Tn for actin and that they function primarily as weakly interacting spacers. The three single-site deletion mutants bound with lower cooperativity than either dAC23 or wild type. Since the single site deletions do not correspond perfectly to one-seventh of TM (39 1/3 residues), the ends may be mismatched relative to each other and to the actin monomer in the filament. This misalignment, if propagated, may result in a long range disorder that could reduce the cooperativity and affinity of binding.

Tropomyosin binding to actin has traditionally been modeled in terms of one TM molecule and the actin subunits along its length in one strand of the actin filament with cooperativity primarily attributed to direct or indirect end-to-end associations between adjacent TM molecules on the filament (37, 38). An alternative interpretation is that when the TM is perfectly matched to the filament and the ends are properly aligned with respect to each other and the monomers in the actin filament,

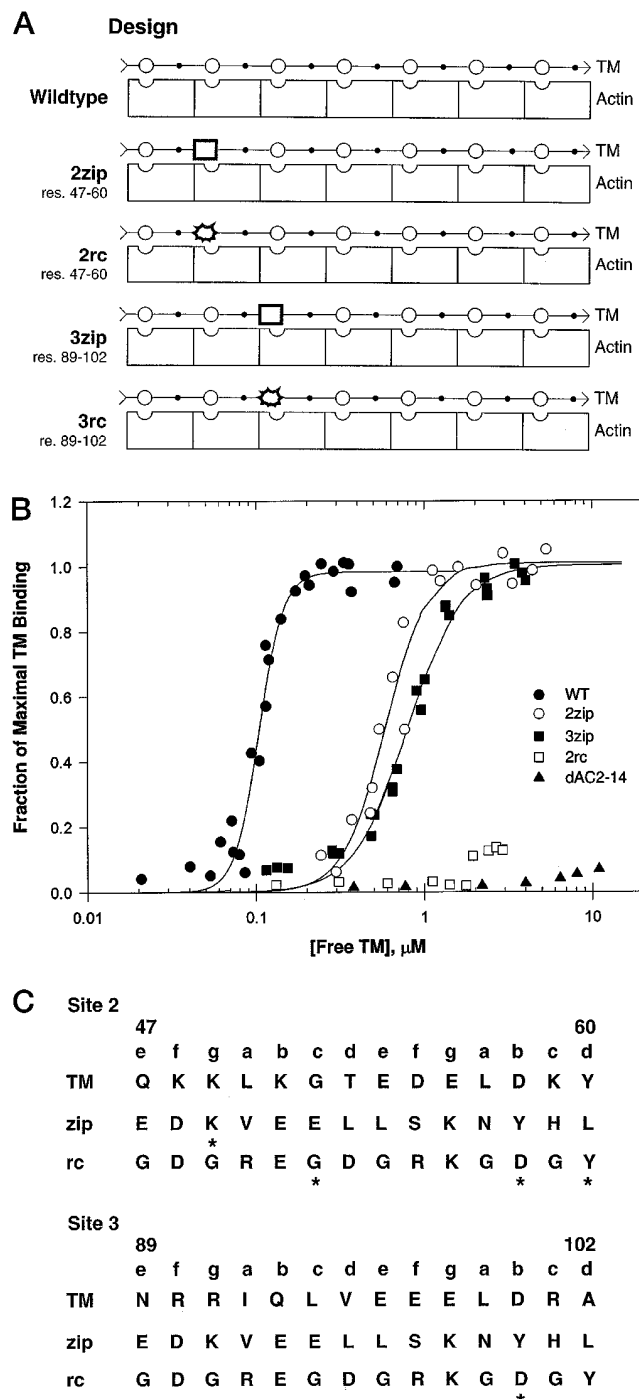


FIG. 2. Replacement of tropomyosin residues with leucine zipper and random coil sequences. *A*, design. The format of the diagram is described in Fig. 1A. Here, 14 residues of site 2 or site 3 were replaced with 14 residues of a GCN4 leucine zipper sequence (Ref. 47, 2zip, 3zip) or 14 residues that should not form an ordered secondary structure (2rc, 3rc). *B*, actin binding of tropomyosin-troponin, with Ca^{2+} . The conditions are the same as in Fig. 1B. dAC2-14 is a mutant in which 14 residues of site 2 (residues 47–60) were deleted. The TM actin binding sites would be out of register with the TM binding sites on actin. The 3rc mutant was not expressed in *E. coli*. The observed binding by 2rc and dAC2-14 is nonsaturating and at the level of trapping. The number of independent data sets used to calculate each curve is as follows: wild type (WT), 3; 2zip, 2; 3zip, 3; 2rc, 1; dAC2-14, 1. The experiments for 2rc and dAC2-14 were carried out in the above buffer with 150 mM NaCl in an attempt to increase actin affinity. The affinity of 2rc for regulated actin was also undetectable at 4 °C. *C*, amino acid sequences of replaced regions and comparison with the tropomyosin sequence. Lower case letters refer to the heptapeptide repeat of amino acids in the coiled coil; a and d are at the interface between the two α -helices (3). Asterisks indicate identities.

the TM molecules may form a seamless cable with, for the purposes of this analysis, an infinite number of equivalent and indistinguishable sites. We consider this model unlikely because it is well established that alterations at either the N or C terminus can profoundly affect cooperative actin binding (28, 29, 35, 36, 39–46, 50). Furthermore, as will now be discussed, changes in internal sequence have small, but significant effects on actin binding.

Sequence Requirements for Actin Binding—Although the N- and C-terminal sequences are primary determinants of actin affinity, small differences have been attributed to alternatively spliced exons. In α -TMs, approximately 2-fold differences in affinity for regulated and unregulated actin can be attributed to the choice of second (2a *versus* 2b, residues 39–80) and sixth exon (6a *versus* 6b, residues 189–214) (43, 47, 48).

To investigate the structural requirements for actin binding in a more fundamental sense, we replaced 14 residues of site 2 (residues 47–60) or site 3 (residues 89–102) with 14 residues of the GCN4 leucine zipper (2zip, 3zip; Ref. 49) or a random coil sequence (2rc, 3rc; Fig. 2A). The GCN4 (zip) sequence shares only one amino acid identity with site 2 and none with site 3 (Fig. 2C). Since the sequence is highly favorable for coiled coil formation, the length and conformation of the 2zip and 3zip should be similar, if not identical, to wild type TM. The random coil sequence (Fig. 2C) was designed with glycines at every second or third residue to prevent formation of secondary structure. The rest of the amino acids were charged in keeping with the highly charged nature of TM. The random coil replacement would interrupt the coiled coil structure of TM. All replacement mutants were expressed well in *E. coli* and could be purified using the normal procedure with the exception of the 3rc, which was not expressed at detectable levels using two different vectors in two different *E. coli* strains. The 2zip and 3zip mutants were similar in stability to wild type, whereas 2rc was less stable (See “Experimental Procedures” and Ref. 25).

Both 2zip and 3zip bound well to regulated actin with affinities similar to that of dAC2 (Fig. 2B, Table I), an effect that is in the same order of magnitude as switching exon 2 or 6 in different TM isoforms (43, 45, 47, 48). Interestingly, both the actin affinity and the cooperativity were reduced. Since the lengths of the 2zip and 3zip should be the same as wild type, the relationship of the ends to each other and to actin should not be altered. This implies that the change in cooperativity relates to the interaction of TM·Tn with actin or to the local sequence and conformation of the TM molecule. It is also possible that the mutation has a long range effect on the ends.

In contrast, 2rc had no detectable affinity for regulated actin in the presence or absence of Ca^{2+} , even at 4 °C, where it was nearly fully helical (Fig. 2B). The 2rc chains are parallel since they could be fully cross-linked via Cys-190. A second mutant where the first 11 residues of the random coil sequence replaced residues 39–49 of site 2 also showed no detectable actin affinity. Fig. 2B shows in addition that deletion of 14 residues of site 2 (dAC2–14) corresponding to one third of a repeat, resulted in loss of actin affinity as predicted by previous work (20).

Conclusions—Essential requirements for TM·Tn binding to actin are that TM be a coiled coil along its entire length and that there be a perfectly integral number of periodic repeats. We suggest that quasiequivalent interactions between TM and each actin monomer along its length cannot account for TM·Tn affinity for actin. The internal repeats may function as weakly interacting spacers to ensure proper alignment of the ends,

critical for cooperative actin binding on the actin filament.

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