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

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Regulated Actin Filament*

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), 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 tropinin (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 coiled 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 Ca2+-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 the sequence of 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 plT11d at the NcoI and BamHI sites and transformed into Escherichia coli strains BL21(DE3) or BL21(DE3)lyS 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): 2zip, 5′-GCCAAGCGCTCCTG-3′; 3zip, 5′-GACCTTCATTCCTCTTTC/CA-GAGCCACCCAGC-3′; 2rc, 5′-GCCAAGCGCTCCTG-3′; 3rc, 5′-GACCTTCATTCCTCTTTC/CA-GAGCCACCCAGC-3′; dAC2–14, 5′-ATCTTTAAGGAGCTCAGCA/GAGCCACCCAGC-3′. The dAC2 mutation was described in Ref. 20. The replacement mutants were made using the following oligonucleotides (antisense sequence, the slash indicates the site of the deletion): Zip, 5′-ATCTTTAAGGAGCTCAGCA/GAGCCACCCAGC-3′; 3zip, 5′-GCCAAGCGCTCCTG/CA-GAGCCACCCAGC-3′; 2rc, 5′-ATCTTTAAGGAGCTCAGCA/GAGCCACCCAGC-3′; 3rc, 5′-GACCTTCATTCCTCTTTC/CA-GAGCCACCCAGC-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): Zip, 5′-ATCTTTAAGGAGCTCAGCA/GAGCCACCCAGC-3′; 3zip, 5′-GCCAAGCGCTCCTG/CA-GAGCCACCCAGC-3′; 2rc, 5′-ATCTTTAAGGAGCTCAGCA/GAGCCACCCAGC-3′; 3rc, 5′-GACCTTCATTCCTCTTTC/CA-GAGCCACCCAGC-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): Zip, 5′-ATCTTTAAGGAGCTCAGCA/GAGCCACCCAGC-3′; 3zip, 5′-GCCAAGCGCTCCTG/CA-GAGCCACCCAGC-3′; 2rc, 5′-ATCTTTAAGGAGCTCAGCA/GAGCCACCCAGC-3′; 3rc, 5′-GACCTTCATTCCTCTTTC/CA-GAGCCACCCAGC-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): Zip, 5′-ATCTTTAAGGAGCTCAGCA/GAGCCACCCAGC-3′; 3zip, 5′-GCCAAGCGCTCCTG/CA-GAGCCACCCAGC-3′; 2rc, 5′-ATCTTTAAGGAGCTCAGCA/GAGCCACCCAGC-3′; 3rc, 5′-GACCTTCATTCCTCTTTC/CA-GAGCCACCCAGC-3′.
Consequently, the deletions do not correspond perfectly to the heptapeptide repeat important for coiled coil formation (2, 3). The deletions were 35 TM repeats contributing in an equivalent way to the overall actin affinity of TM two- to seven-fold, depending on the ionic strength of dAC2, 42 amino acids, residues 47–88) reduced the actin affinity, 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 Muclachlan and Stewart model (78% residues; Ref. 5), and the same as in the Phillips proposal (6). Surprisingly, the $K_{\text{app}}$ of dAC23 TM-Tn for actin was reduced only 2-fold compared with wild-type TM, and the cooperativity was essentially unchanged. The $K_{\text{app}}$ of dAC2 was considerably higher than either dAC3 mutant, suggesting that 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. Considering the helical (azimuthal) position on the $\alpha$-helix as well as the linear (supercell) position of each amino acid, Phillips (6) postulated that the repeats were 42 residues (e.g. 35 residues (site 3), with an average of 39½ amino acids.

the data in the binding isotherms in Figs. 1B and Table I. The data were analyzed using the Hill equation as well as a linear lattice model (37, 38). The $K_{\text{app}}$ 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 ($K_a$) and cooperativity ($\alpha$ or $\gamma$). 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.

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 $K_{\text{app}}$ of dAC23 is 62% of wild type (versus 46% when calculated in terms of molecular weight), while the $K_a$ 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)

### Table I

| TM       | $K_{\text{app}}$ | Hill coefficient | $K_a$ | $\gamma$ | $\gamma K_a$ |
|----------|------------------|------------------|-------|---------|-------------|
| Wild type | $10^{-6}$ M$^{-1}$ | 6.0 ± 1.6        | 4.2 ± 1.0 | 227 ± 57 | 9.5 ± 3.3 |
| dAC2     | $10^{-6}$ M$^{-1}$ | 2.5 ± 0.4        | 3.1 ± 0.3 | 40 ± 4   | 1.2 ± 0.2  |
| dAC2-14  | -0.1             | 2.7 ± 0.7        | 0.94 ± 0.2 | 52 ± 11  | 0.49 ± 0.1 |
| dAC3-35  | 0.53 ± 0.06      | 2.5 ± 0.6        | 1.1 ± 0.2 | 39 ± 6   | 0.44 ± 0.1 |
| dAC3-42  | 0.48 ± 0.04      | 5.3 ± 1.5        | 2.8 ± 0.7 | 151 ± 36 | 4.2 ± 0.4  |
| dAC23    | 4.3 ± 0.4        | 3.4 ± 1.2        | 1.8 ± 0.6 | 92 ± 30  | 1.6 ± 0.4  |
| 2zip     | 1.7 ± 0.2        | 2.7 ± 0.5        | 2.1 ± 0.2 | 55 ± 18  | 1.2 ± 0.2  |
| 3zip     | 1.3 ± 0.1        | -0.1             | 0.1     | 0.1      | 0.1        |

### 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 Muclachlan and Stewart (5).

2 N. J. Greenfield, unpublished results.
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 dAC2 or wild type. Since the single site deletions do not correspond perfectly to one-seventh of TM (39\(\frac{1}{7}\) 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 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 \(\alpha\) and \(\beta\)-actin binding sites postulated by McLachlan and Stewart (5). Tropinin 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 \(\mu\)M chicken pectoral actin; chicken pectoral Tn and TM in a 1:2.1 molar ratio, 0 to 2–15 \(\mu\)M, 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.

**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 \(\alpha\) and \(\beta\)-actin binding sites postulated by McLachlan and Stewart (5). Tropinin 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 \(\mu\)M chicken pectoral actin; chicken pectoral Tn and TM in a 1:2.1 molar ratio, 0 to 2–15 \(\mu\)M, 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.

**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. 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 \(\alpha\)-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 (2zip) 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 Ca2+, even at 4°C, where it was nearly fully helical (Fig. 2B). The 2rc chains are parallel since 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 quasi-equivalent 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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