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What makes tropomyosin an actin binding protein? A perspective

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Abstract

Tropomyosin is a two-chained α -helical coiled coil that binds along the length of the actin filament and regulates its function. The paper addresses the question of how a “simple” coiled coil sequence encodes the information for binding and regulating the actin filament, its universal target. Determination of the tropomyosin sequence confirmed Crick's predicted heptapeptide repeat of hydrophobic interface residues and revealed additional features that have shown to be important for its function: a sevenfold periodicity predicted to correspond to actin binding sites and interruptions of the interface with destabilizing residues, such as Ala. Evidence from published work is summarized, leading to the proposal of a paradigm that binding of tropomyosin to the actin filament requires local instability as well as regions of flexibility. The flexibility derives from bends and local unfolding at regions with a destabilized coiled coil interface, as well as from the dynamic end-to-end complex. The features are required for tropomyosin to assume the form of the actin helix, and to bind to actin monomers along its length. The requirement of instability/flexibility for binding may be generalized for the binding of other coiled coils to their targets.

Keywords

coiled coil protein; Ala clusters; actin filament; thin filament regulation; disordered protein

1. Introduction

Tropomyosin is a “simple” α -helical coiled coil that associates end-to-end and cooperatively binds along the length of the actin filament, conforming to the filament helix; for reviews see (Brown and Cohen, 2005; Hitchcock-DeGregori, 2008; Lehman and Craig, 2008). Tropomyosin, stiffens the actin filament, and in doing so inhibits the action of severing and crosslinking proteins, and the formation of branches nucleated by the Arp2/3 complex.

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Together with its roles in regulating caldesmon function and polymerization dynamics of the filament ends alone and with tropomodulin and formin, tropomyosin controls cellular migration and is an obligatory component of the contractile ring that forms for cytokinesis. Tropomyosin also cooperatively regulates motile and contractile functions via interaction with myosin and troponin in striated muscles, reviewed in (Gunning et al., 2008).

What makes tropomyosin an actin binding protein? The observed $<\mu\text{M}$ affinity is an apparent affinity that depends on cooperative binding; the affinity of a single molecule is in the mM range (Landis et al., 1999; Wegner and Walsh, 1981). How is binding specificity encoded in the sequence? How does a simple α -helical coiled-coil protein, such as tropomyosin, bind to a target? Here we address this question, summarizing our own work as well as that of others and lead to the proposal of a paradigm: binding of a coiled coil to its target requires regions of local instability or disorder that allow surface residues to assume an optimal conformation for binding its target(s). In the case of tropomyosins, the same properties allow the coiled coil to bend and conform to the filament helix ((Brown et al., 2001). We will focus on tropomyosin binding to its universal target, the actin filament. The context will be vertebrate striated muscle tropomyosin since most is known about the structure and function of this form. However, the generalizations can be extended to most, if not all tropomyosins.

The tropomyosin coiled coil binds end-to-end to form continuous strands along both sides of the actin filament, such that one molecule spans the length of seven actin monomers. In the bound state, the tropomyosin supercoil conforms to the helical filament (Holmes and Lehman, 2008; Lehman and Craig, 2008). The molecular ends of tropomyosin form a flexible four-helix intermolecular complex between the first and last eleven residues that is required for cooperative actin binding (Greenfield et al., 2006). How the initial molecule(s) bind to actin is unknown, but binding increases the effective local concentration of weakly-associated molecules.

Our understanding of where tropomyosin binds to the actin filament comes from EM reconstructions (most helically averaged) and fiber diffraction studies coupled with molecular modeling; there is no high resolution structure (Lehman et al., 2000; Lorenz et al., 1995). It is well documented that tropomyosin (with troponin) can assume three average positions on the actin filament that are proposed to correspond to three kinetic states of the regulated thin filament (Lehman and Craig, 2008; Xu et al., 1999). Tropomyosin's flexible end-to-end complex and weak association with individual monomers underlie its cooperative regulatory properties (Geeves and Lehrer, 1994).

2. Periodic sequence repeats, and variations

Clues about tropomyosin's binding sites are in the amino acid sequence. The heptapeptide repeat of hydrophobic residues that Crick deduced from the helical diffraction pattern (Crick, 1953) was confirmed when the tropomyosin amino acid sequence was available (the first coiled coil sequence) (McLachlan et al., 1975; Parry, 1975; Stone and Smillie, 1978). Analysis of tropomyosin's sequence revealed features, in addition to Crick's predicted heptapeptide repeat, that more recently have been proven to be of structural significance and to be important for tropomyosin's function on actin. One is a sevenfold (or fourteen-fold, " α and β sites") repeat postulated to represent actin binding sites, since one tropomyosin is the length of seven actin monomers in the filament (McLachlan and Stewart, 1976; McLachlan et al., 1975; Parry, 1975). An analysis that took into account the azimuthal positions of the residues on the coiled coil identified a sevenfold repeat of seven charged and hydrophobic surface residues that correspond approximately to the McLachlan and Stewart α -sites, referred to here as "consensus" residues and illustrated in cyan in Figure 1 (Phillips, 1986).

Their functional relevance is inferred by the clustering of disease-causing mutations in these regions.

2.1 Tropomyosin's binding to the actin filament: Relationship of the periodic repeats to actin subunits

The molecular reconstructions of the regulated thin filament do not define the relationship of tropomyosin's periodic repeats to the actin subunits because the ends of tropomyosin cannot be identified. Determination of the structure of the complex between the molecular ends of tropomyosin, however, did answer a major unresolved question with regard to the relationship of the periodic sites in sequential tropomyosin molecules to actin: they have equivalent relationships to actin subunits in the filament (Figure 2).

In the end-to-end complex, the C terminus splays apart to form a cleft where the N terminus binds and overlaps eleven residues of the C terminus, as discussed in below in section 2.4 (Greenfield et al., 2006). The planes of the two coiled coils in the complex are perpendicular to each other (Figure 2b). The pitch of the tropomyosin supercoil is variable, such that there are ~2.75 turns of the supercoil per molecule (5.5 half turns), not 3.0 as supposed in earlier models (Phillips, 1986; Whitby and Phillips, 2000). The 90° phase shift in orientation “corrects” for the non-integral number of repeats so that the surface residues of sequential tropomyosins have nearly equivalent orientations relative to actin (Figure 2c). If the planes of the two coiled coils in the complex were parallel, or if the number of half-turns of the supercoil were integral with a 90° phase shift (Whitby and Phillips, 2000), only alternating molecules would have similar relationships to actin (Greenfield et al., 2006).

Careful examination of the positions of the repeats shows that the spacing is not perfectly regular (Figure 1). The variations in the supercoil pitch and within the coiled coil (Brown et al., 2005; Brown et al., 2001; Li et al., 2002; Minakata et al., 2008; Nitanaï et al., 2007; Whitby and Phillips, 2000) (discussed below in sections 2.2-2.4), and especially the conformational flexibility in the intermolecular junction (Greenfield et al., 2006) allow tropomyosin molecules to conform to the actin structure along the length of the filament and to move on the actin filament in response to myosin and troponin.

2.2 Interruptions in tropomyosins's canonical coiled coil: Ala clusters

Also noticed early, but appreciated more recently with the availability of high resolution structures, is an irregular interruption of the canonical hydrophobic interface residues at the *a* and *d* positions by destabilizing residues, such as Ala, referred to as “Ala clusters,” (Brown et al., 2001; Kwok and Hodges, 2004; McLachlan et al., 1975). These are highlighted in magenta in Figure 1. An understanding of the significance of the Ala clusters came with the atomic resolution structure of the first 80 residues of tropomyosin (Brown et al., 2001). The interhelical distance is ~2 Å smaller at the Ala clusters than in other regions, and the interface is less well-packed than in a canonical coiled coil. In some instances the chains are staggered, resulting in a bend in the molecule, thereby introducing asymmetry into the structure (Brown, 2006). Similar deviations from canonical coiled-coil structure have been observed in other regions of tropomyosin (Brown et al., 2005; Li et al., 2002; Minakata et al., 2008; Murakami et al., 2008; Nitanaï et al., 2007). Such variations can have global effects on the structure, as have been shown for skips and stutters in the underlying heptad repeat (Brown et al., 1996; Strelkov et al., 2002).

2.3 Importance of the destabilizing Ala clusters for actin binding

Tropomyosin is unusual in being an uninterrupted coiled coil. Other long coiled coils frequently have “skips” or “stutters,” interruptions in the heptapeptide repeat that destabilize the coiled coil (Brown et al., 1996; Parry et al., 2008). Yeast tropomyosin lacks Ala clusters,

but has such an interruption (Liu and Bretscher, 1989). We have postulated that the Ala clusters serve to destabilize the coiled coil and promote actin binding by increasing affinity at individual sites and giving the molecular “flexibility” (an overused term) for tropomyosin to conform to the filament's helix.

Mutagenesis experiments showed that local destabilization of the coiled coil interface is a crucial parameter for actin binding. Replacement of Ala clusters in period 2 or period 5 (Figure 3a) with canonical hydrophobic residues (Leu, Val) stabilizes the molecule, increases the overall T_m by 13–37 °C, depending on the mutant and conditions, and results in loss of measurable actin affinity (Singh and Hitchcock-DeGregori, 2003; Singh and Hitchcock-DeGregori, 2006). The requirement for actin binding is destabilization rather than a specificity for Ala. Replacement of the Ala clusters with Asn and Gln, polar residues that destabilize the coiled coil but do not decrease the interhelical distance (Kwok and Hodges, 2004) results in near wildtype stability, actin affinity and regulatory function (Singh and Hitchcock-DeGregori, 2003; Singh and Hitchcock-DeGregori, 2006; Singh and Hitchcock-DeGregori, 2007). Major destabilization of the coiled coil by replacement of a sequence with a non-coiled coil sequence, destroys actin binding (Hitchcock-DeGregori and An, 1996).

2.4 Other non-canonical interface regions of tropomyosin

In addition to the Ala clusters, there are two global bends evident in the 7 Å structure of full-length tropomyosin (Whitby and Phillips, 2000) that are explained by examination of atomic resolution structures. The first bend is at D137 (in a *d* position), with a neighboring interface Ala (in an *a* position) (Brown et al., 2005). Stabilization by an Asp-to-Leu mutation affects Ca^{2+} -dependent regulation of the actomyosin ATPase with troponin without affecting actin affinity (Sumida et al., 2008). The second bend is where Y214, E218 and Y221, at *d*, *a*, *d* interface positions results in a poorly-packed interface and increased interhelical distance (Minakata et al., 2008; Nitanaï et al., 2007). Both D137 and E218 are highly-conserved residues.

A third location with a non-canonical interface is at the C terminus. Q263 (at a *d* position) and Y267 (at an *a* position) are poorly-packed and conformationally flexible in solution. The atypical coiled coil sequence drives the two helices to assume a parallel arrangement (Figure 2a) (Greenfield et al., 2002; Greenfield et al., 2006). The structure is reminiscent of a conserved domain of the intermediate filament protein, vimentin, that contains a stutter where the chains become almost parallel because of local unwinding of the supercoil (Strelkov et al., 2002). The flexibility at Q263 allows the ends to splay apart and bind to the N terminus to form a dynamic complex (Figure 2b) and to bind to itself in an antiparallel fashion in crystal structures (Li et al., 2002; Minakata et al., 2008; Nitanaï et al., 2007). The conformational dynamics are essential for function since mutation of Q263 to the canonical interface residue, Leu, stabilizes the C terminus and prevents it from binding the N terminus and forming a complex with troponin T (Greenfield et al., 2002).

3 Are the periodic repeats actin binding sites?

3.1 Dissection of a periodic repeat: A dual requirement for specific surface residues and an unstable interface

Evidence has accumulated that the observed periodic repeats of surface residues do represent actin binding sites, but that the periods contribute in different ways to tropomyosin's binding and regulatory functions. Analysis of a series of deletion and substitution mutants showed that the presence of the ends and period 5 are required for cooperative actin binding. Modifications of other periods have smaller effects on actin affinity but influence regulatory function with troponin and cooperative activation by

myosin (Hitchcock-DeGregori et al., 2001; Hitchcock-DeGregori et al., 2002; Kawai, 2009; Landis et al., 1999; Landis et al., 1997; Novy et al., 1993; Sakuma et al., 2006; Singh and Hitchcock-DeGregori, 2007). Based on these analyses, we have proposed that period 1 (included in the overlap complex with the C terminus) and period 5 are “primary,” and the remaining five are “secondary” actin binding sites.

Given the requirement for interface instability, a non-specific effect, it was necessary to distinguish the contribution of surface residues from that of interface stability to actin binding. We carried out such an analysis in the context of period 5 (residues 165-185, Figure 3b). Period 5 is the only period aside from period 1 where the Ala cluster is within the proposed actin binding site. Period 5 is critical for function. Deletion results in loss of function and a mutant in which a GCN4 leucine zipper sequence was substituted for period 5 (the region in the box in Figure 3b) is much more stable than wildtype and fails to bind actin (Hitchcock-DeGregori et al., 2002).

Analysis of a series of mutants showed that restoration of actin binding requires the wildtype surface residues as well as an unstable interface (Singh and Hitchcock-DeGregori, 2006). This was the first evidence that the sequence of a periodic repeat is crucial for actin binding. The conclusion was supported by additional experiments in which period 5 was replaced by period 1 or period 2 sequence (Figure 3c). The mutants bound actin as long as the region with the “consensus” surface residues (shown in cyan) were “in phase” (the correct position in the periodic repeat, red boxes) and included an Ala cluster (Singh and Hitchcock-DeGregori, 2007). The work provided the first experimental support that the periodic repeats are quasi-equivalent actin binding sites. Thus, there is a dual requirement for specific surface residues and an unstable interface, requirements met in periods 1 and 5, the “primary” sites. Those that do not have an “embedded” Ala cluster contribute less to overall affinity and are referred to as “secondary” sites.

3.2 Engineering increased actin affinity in a periodic repeat: Contributions to affinity and cooperativity of binding

Periods 2 and 3 contribute the least to the overall actin affinity (Hitchcock-DeGregori and Varnell, 1990; Hitchcock-DeGregori and An, 1996; Hitchcock-DeGregori et al., 2002). If the primary reason for their “secondary” status is the absence of a destabilizing interface, we reasoned that engineering an embedded Ala cluster into these sites, thereby satisfying the dual requirement of sequence and a destabilized interface, should increase actin affinity (Singh and Hitchcock-DeGregori, 2009). Introduction of an Ala cluster into period 2 increased actin affinity, supporting the hypothesis. A comparable mutation in period 3 increased the cooperativity of actin binding with little effect on the overall actin affinity, as did replacement of the period 3 sequence with the period 5 sequence, a “primary” site that includes an Ala cluster (Figure 3d). The results indicate those internal periods, not just the ends, contribute to the cooperativity of binding.

4. Conclusions

4.1 Characteristics of an actin binding site

It is well established that regions of tropomyosin vary in stability and some are at least partially unfolded at physiological temperature (Hitchcock-DeGregori et al., 2002; Hodges et al., 2009; Ishii and Lehrer, 1985; Ishii and Lehrer, 1990; Potekhin and Privalov, 1982). Actin binding stabilizes tropomyosin (Levitsky et al., 2000). Here we have summarized our work showing that local instability at the periodic repeats is required for cooperative actin binding. Actin binding locally stabilizes these regions (Singh and Hitchcock-DeGregori, 2009).

The affinity of each binding site for actin is weak, but specific, as defined by the ability to discriminate between alternate ligands and targets. Actin binding is predominantly electrostatic; electrostatic interactions are weak but stereo-chemically specific, without necessitating a “lock and key” type of stereo-specific complementarity. There is also a hydrophobic component of binding, as illustrated by the classic cold-sensitivity of tropomyosin binding to actin, used to advantage for actin purification. We suggest that the primary sites (at the end and near the middle of tropomyosin) initially bind to actin and position secondary sites to where they can bind the filament. Critical to this is the “flexibility” of the recognition domains and their ability to dynamically orient for optimal binding to the target. Conformational disorder or entropy may allow tropomyosin to bind to different sites on actin, and bend as necessary to conform to the helical filament. Since tropomyosin moves on the surface of the actin filament in response to myosin, troponin and Ca^{2+} , it must be able to recognize different regions of the actin monomer. While the less stable regions of tropomyosin may not be as disordered as domains in intrinsically disordered proteins (Tomba et al., 2009), the conformational entropy may be important for molecular recognition, as it is for proteins such as calmodulin that can bind to a variety of targets (Frederick et al., 2007).

4.2 Binding of tropomyosin to the helical actin filament

Tropomyosin is no ordinary coiled coil: it is exquisitely designed for its roles in actin filament regulation. The specific design is evident in its sevenfold periodic repeat that corresponds to seven actin monomers along its length (in high molecular weight, “long” forms; four to six actin monomers in other forms). Interruptions in the canonical coiled-coil interface that result in bends and local unfolding, the variable supercoil pitch and the flexible end-to-end junction allow tropomyosin to follow the contour of the actin filament helix and assume different positions on its surface. That is, tropomyosin's functions are intrinsic in its design, as in globular proteins, despite its simple coiled-coil structure. Mutations, whether by design or genetic disease, that have global effects on the structure and stability alter tropomyosin function, as they do in globular proteins.

Our understanding of the requirement for regions of coiled coil instability for bending and molecular recognition of actin may seem to differ conceptually from the “Gestalt-binding” model in which the form of the tropomyosin is designed to match that of the actin filament (Holmes and Lehman, 2008). Tropomyosin, like any folded protein, has a shape while maintaining a dynamic “persona”. We suggest that the sequence and the supercoil define a bias toward the right-handed actin filament helix, but that unbound tropomyosin assumes multiple forms. The conformational dynamics observed in solution, the presence of partially unfolded regions at physiological temperature, and structural variations between different molecules in the unit cells of crystal structures all suggest the possibility of many forms. A recent study showing that the overall molecule is semi-rigid, with variations in coiled-coil radius and local curvature and flexibility (Li et al., 2009) is consistent with this view and expected based on the sequence, structures and many solution and mutagenesis studies. The observed global stiffening of tropomyosin molecules by mutations that grossly increase stability (Li et al., 2009; Singh and Hitchcock-DeGregori, 2003) supports the hypothesis that flexible regions allow tropomyosin to assume the shape of the actin helix. The dynamic intermolecular junction results in a continuous flexible cable of tropomyosin molecules along the length of the helical filament that may be important for cooperative azimuthal movements in response to regulatory signals.

The sevenfold repeat is intrinsic to tropomyosin's design to bind along seven actin monomers in the filament. The requirement for a specific pattern of charged and hydrophobic surface residues (Singh and Hitchcock-DeGregori, 2006; Singh and Hitchcock-DeGregori, 2007) that complements an expected binding surface on actin (Brown et al.,

2005) illustrates that specificity goes beyond global shape. Strong stereo-specific binding of the lock-and-key type, however, is not involved. It has been suggested a binding site in a relatively unstructured state can bind weakly to its target at a greater distance than is possible with a fully-folded protein, termed the “fly-casting mechanism” (Shoemaker et al., 2000). Such weak binding to different sites on individual actin subunits may underlie tropomyosin's cooperative switching between regulatory states on the filament.

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Figure 1. Structure of striated muscle α -tropomyosin

The side chains of the alanine clusters (magenta) and consensus residues in proposed actin binding sites (cyan) (Phillips, 1986) are illustrated on a ribbon model of the 7 Angstrom structure of tropomyosin (Whitby and Phillips, 2000). The numbers correspond to the seven periodic repeats, 1-7. Note that the spacing of the periodic repeats is not perfectly regular. The Ala clusters (Brown et al., 2001) are positioned within consensus residues only in periods 1 and 5, but do not have a regular relationship to the other periods. Modified from (Singh and Hitchcock-DeGregori, 2006).

Figure 2. Solution structure of peptide models of the striated α -tropomyosin junctional complex and implications for actin binding

Modified from (Greenfield et al., 2006). a. The conformation of the C-terminal domain in complex with the N-terminal domain (cyan) compared to unbound C-terminal domain (brown). In the free form the two chains are almost parallel. Upon complex formation the chains splay apart following residue I270. b. Structure of the junctional complex between the C-terminal domain (cyan) and N-terminal domain (brown). The figure shows the overlay of two ribbon models of the complex to illustrate the maximal variation between the calculated structures due to the flexibility of the complex interface. c. Top: A model of the junctional complex was modeled into the 7 Angstrom structure of tropomyosin (Whitby and Phillips, 2000), joining two full-length tropomyosin molecules (Greenfield et al., 2009). The arrow indicates the junction; the side chains of consensus residues are shown in black. The orientations of the consensus residues have similar azimuthal relationships on sequential tropomyosin molecules. This point is emphasized in a model showing only one of the seven consensus residues (middle). Bottom: The model of the joined tropomyosin molecules was docked on a model of the actin filament (tan). The axial position is completely arbitrary, but K238 of actin (red) serves as a position marker on the actin monomers. The image shows the comparable relationship of the periods on sequential tropomyosin molecules to actin subunits in the filament. This would not be the case if the planes of the coiled coils in the junctional complex were parallel.

Figure 3. Model of tropomyosin showing consensus residues and Ala clusters highlighting regions selected for mutagenesis

a. The Ala clusters in period 2 and period 5 are in red boxes. Changing the alanines (magenta) into canonical interface residues (LVL) resulted in loss of function; mutation to QNQ had little effect on function or stability (Singh and Hitchcock-DeGregori, 2003; Singh and Hitchcock-DeGregori, 2006). b. Period 5, boxed in red, contains an Ala cluster within the region of the consensus residues. Extensive mutagenesis showed that both the surface consensus residues and a destabilizing interface are required for tropomyosin function (Singh and Hitchcock-DeGregori, 2006). c. Diagram illustrating the replacement mutants. Period 5 (hatched a red box) was replaced with period 1 (solid red box), period 2 with the consensus residues (solid red box) or the Ala cluster C-terminal to the period 2 consensus residues that contains an incomplete consensus sequence (magenta, hatched box). Function was retained only when period 5 was replaced with sequence that contained both consensus residues and a destabilized interface (Singh and Hitchcock-DeGregori, 2007). d. Diagram illustrating replacement of period 3 with period 5 sequence. Replacement of period 3 that lacks an Ala cluster, with the period 5 sequence increased the cooperativity of binding (Singh and Hitchcock-DeGregori, 2009). Mutations to destabilize period 2 and period 3 consensus regions increased actin affinity and/or the cooperativity of binding.