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Structural determinants of cooperativity in acto-myosin interactions★✪

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Regulation of muscle contraction is a very cooperative process. The presence of tropomyosin on the thin filament is both necessary and sufficient for cooperativity to occur. Data recently obtained with various tropomyosin isoforms and mutants help us to understand better the structural requirements in the thin filament for cooperative protein interactions. Forming an end-to-end overlap between neighboring tropomyosin molecules is not necessary for the cooperativity of the thin filament activation. When direct contacts between tropomyosin molecules are disrupted, the conformational changes in the filament are most probably transmitted cooperatively through actin subunits, although the exact nature of these changes is not known. The function of tropomyosin ends, alternatively expressed in various isoforms, is to confer specific actin affinity. Tropomyosin's affinity or actin is directly related to the size of the apparent cooperative unit defined as the number of actin subunits turned into the active state by binding of one myosin head. Inner sequences of tropomyosin, particularly actin-binding periods 3 to 5, play crucial role in myosin-induced activation of the thin filament. A plausible mechanism of tropomyosin function in this process is that inner tropomyosin regions are either specifically recognized by myosin or they define the right actin conformation required for tropomyosin movement from its blocking position.

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Abbreviations: TM, tropomyosin; Tn, troponin; S1, myosin subfragment 1.

Cyclic interactions between actin thin filaments and myosin heads protruding from the thick filaments, powered by actin-activated ATP hydrolysis on the myosin head, underlie muscle contraction as well as certain forms of non-muscle cells motility. The complex processes involved in the regulation of the interactions between actin and myosin have been studied using biochemical, physiological as well as structural methods. Although each of the approaches emphasizes different aspects of the regulation, all of those studies point to cooperativity as a central feature of the regulation of contraction. Tropomyosin (TM) is the regulatory protein that has been proved to be necessary and sufficient for cooperativity, however, the molecular source of the cooperativity is still a matter of dispute.

TROPOMYOSIN STRUCTURE

Tropomyosin is an integral component of actin thin filament found in all eukaryotic cells. It is an elongated coiled-coil, made of a homodimer or a heterodimer of α-helical chains that binds longitudinally along both grooves of the actin filament (O'Brien *et al.*, 1971; Milligan *et al.*, 1990). All native tropomyosins polymerize head-to-tail forming an overlap that involves about nine amino acids from the C- and N-termini of the neighboring molecules (McLachlan & Steward, 1975). TM isoforms differ in length. The muscle isoforms span the length of seven actin subunits, whereas vertebrate non-muscle cells contain numerous isoforms which are seven or six actins long (Less-Miller & Helfman, 1991). Two tropomyosins expressed in yeast span only five or four actin subunits and are the shortest isoforms known (Drees *et al.*, 1995; Liu & Bretscher, 1989). Tropomyosin amino-acid sequence is characterized by two patterns of periodicities. Seven-residue repeats, in which hydrophobic residues are located in the first and fourth positions, form an interface between helices of two tropomyosin chains and stabilize the coiled-coil structure (McLachlan & Steward, 1975). The second type of periodicity is represented by quasi-equivalent repeats of 40 amino acids ascribed to periodic actin binding sites (McLachlan & Steward, 1976; Philips *et al.*, 1986).

Tropomyosin is found in a large diversity of isoforms. The diversity is generated by different genes (four in vertebrates), alternative promoter selection and alternative splicing of the RNA transcript. The alternative regions comprise N- and C-terminal segments, encoded respectively by exons 1 and 9, and two inner sequences encoded by alternative exons 2 and 6. The sequence differences determine TM functions such as actin affinity, interaction with troponin (Tn), activation of actomyosin Mg-ATPase, and stabilization of actin filament (Lees-Miller & Helfman, 1991; Pittenger *et al.*, 1994; Perry, 2001).

REGULATION OF ACTO-MYOSIN INTERACTIONS

Regulation of interactions between actin and myosin is best understood in striated muscle. In skeletal and cardiac muscle each TM binds one Tn complex made of three subunits – troponin T, troponin C and troponin I. Troponin T is an elongated tropomyosin-binding molecule which bridges and apparently strenghtens the head-to-tail overlap between neighboring tropomyosins. The presence of troponin C confers Ca^{2+}-sensitivity to the regulation of acto-myosin interactions. Troponin I binds actin and all troponin subunits forming extensive inter-protein contacts. The strength of some of the contacts is regulated by binding of Ca^{2+} to troponin C (recently reviewed by Gordon *et al.*, 2000).

A widely accepted model of the striated muscle regulation, based on kinetic studies, describes the thin filament in terms of an equilibrium between three functional states set up by regulatory components (McKillop & Geeves, 1993). In the absence of Ca^{2+} the thin

filament is mostly in the "blocked" state, in which myosin does not bind to actin (except for weak electrostatic interactions) and therefore the actomyosin Mg-ATPase is off. Ca^{2+} binding to troponin C shifts the equilibrium towards the "closed" state, which allows weak myosin binding and low Mg-ATPase activity. Transition of the filament into the fully active "open" state is induced by myosin heads strongly bound to actin.

The three functional states correspond to structural states characterized by three different tropomyosin positions on the thin filament (Fig. 1). X-Ray diffraction on muscle fibers and helical three-dimensional reconstructions of electromicroscopic images of the regulated filaments revealed that in low Ca^{2+} tropomyosin strings occupy a position on the outer actin domain and sterically block all myosin-binding sites on actin. Ca^{2+} binding to troponin induces conformational changes causing an azimuthal shift of tropomyosin by 25^{o}. This uncovers most but not all myosin sites on actin allowing weak myosin binding (Lehman *et al.*, 1994; Poole *et al.*, 1995; Vibert *et al.*, 1997; Xu *et al.*, 1999). In the third, "open" state TM shifts further towards actin inner domain and exposes all myosin contact

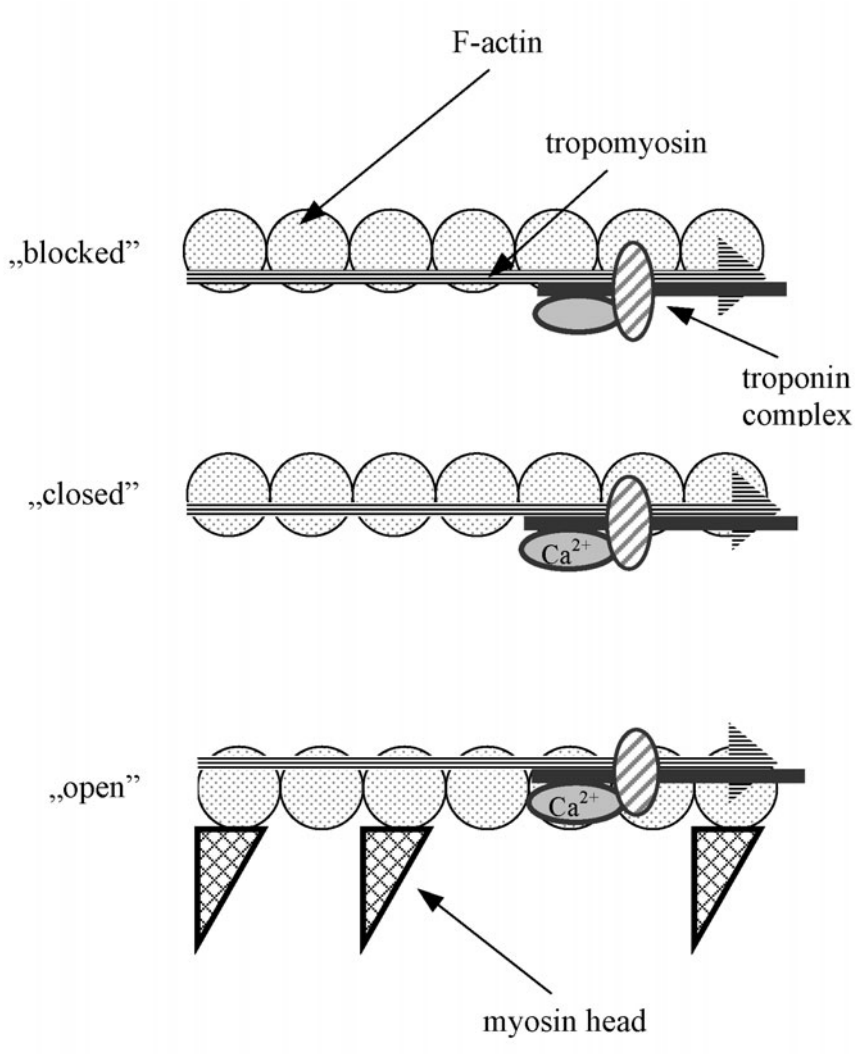

Figure 1. Schematic representation of the thin filament's activation states.

Transition from "blocked" to "closed" state upon binding of Ca^{2+} to troponin C (gray ellipse) is accompanied by an azimuthal shift of tropomyosin (stripped arrow) on actin (dotted circles). Fully active state ("open state") and further tropomyosin shift is induced by myosin heads (hatched triangles) strongly bound to actin.

sites on actin. This state was observed in the presence of myosin heads strongly bound to actin (Vibert *et al.*, 1997; Craig & Lehman, 2001).

In smooth muscle and non-muscle cells, which lack the troponin complex, the actomyosin ATPase is regulated by Ca^{2+}-dependent phosphorylation of myosin light chain and other regulatory proteins, e.g. caldesmon (Sellers & Adelstein, 1986). In thin filaments reconstituted *in vitro* in the absence of troponin, tropomyosin is in equilibrium between the "closed" and "open" states (Lehman *et al.*, 2000). Thus, tropomyosin, common in muscle and non-muscle cells, may regulate acto-myosin interactions *via* different mechanisms.

STRUCTURAL DETERMINANTS OF COOPERATIVITY

In the presence of tropomyosin the interactions between contractile proteins are very cooperative. Cooperativity is the feature characteristic to tropomyosin binding to actin, myosin S1 (myosin proteolytic subfragment comprising head region) binding to the thin filament, especially in the absence of Ca^{2+}, Ca^{2+}-activation of contraction, and activation of the thin filament by myosin S1 (reviewed in Tobacman, 1996; Gordon *et al.*, 2000). Structural and functional diversity among TM isoforms raises a question about the structural basis for cooperativity.

The first factor that was taken into consideration was the end-to-end overlap between TM molecules. In the early model of Hill and coworkers (Hill *et al.*, 1980), cooperativity is a consequence of a concerted change within the functional unit of 7 actin–TM–Tn. Nearest neighbor interactions between the units due to TM–TM overlap determine cooperative transmission of conformational changes along the filament. A more recent model, proposed by Geeves & Lehrer (1994), differs from the Hill's model in that the cooperative unit can be either longer or shorter than seven actins. In this model the size of the apparent cooperative unit is defined as the average number of actin subunits trapped in the open state by one myosin head bound.

The results obtained by Lehrer and colleagues (1997) point to the strength of end-to-end interactions as the major determinant of cooperativity. The authors compared the cooperativities of the closed-to-open transition of actin complexed with one of the three tropomyosin isoforms — gizzard TM and 5aTM from fibroblasts, which differ at the N-terminus but share the same sequence of the 27 amino-acid C-terminal segment, and striated TM with the C-terminal sequence unique for this isoform. The cooperative unit size, calculated from fluorescence changes of pyrene-labeled TM in function of myosin S1 bound to actin, was similar for smooth muscle TM and non-muscle 5aTM. In the presence of striated muscle TM the size of the cooperative unit was about twofold smaller. The sequence of the C-terminus must therefore determine the strength of TM–TM interactions and be responsible for cooperativity, the stronger interactions the larger is the actin segment remaining under control of one tropomyosin molecule.

However, data obtained with N- and C-terminally truncated tropomyosins raise doubts as to whether the end-to-end interactions are the main determinant of cooperativity. Truncation of tropomyosin ends drastically changes properties of the protein. Removal of nine to eleven amino acids from the C-terminus strongly reduces TM affinity to actin alone (Dąbrowska *et al.*, 1983; Hammel & Hitchcock-DeGregori, 1996). In the presence of Tn (Ca^{2+}) the actin affinity of C-terminally truncated tropomyosin increases (Dąbrowska *et al.*, 1983; Pan *et al.*, 1989; Butters *et al.*, 1993; Hammel & Hitchcock-DeGregori, 1996), whereas TM with the N-terminus removed does not bind in either conditions (Cho *et al.*, 1990; Moraczewska & Hitchcock-DeGregori, 2000).

Despite the reduced actin affinity, truncated tropomyosins could still be used in an analysis of the cooperativity of the closed-to-open transition. The approach was based on the observation of Barbara Eaton (1976) that myosin heads are able to induce actin binding of tropomyosin in the tight TM-binding "open" state, even under conditions in which TM alone does not bind to actin. Two tropomyosin isoforms – striated muscle αTM and non-muscle TM2 – were mutated to produce truncated forms depleted of nine amino-acid residues at the N-, C-, or both ends (Moraczewska & Hitchcock-DeGregori, 2000). The isoforms were identical except for the sequence of 27 C-terminal amino acids that were either striated muscle specific, encoded by exon 9a (TM9a), or constitutive, encoded by exon 9d (TM9d) and expressed in smooth muscle and many non-muscle isoforms. The transition of the thin filament from the "closed" to the "open" state was monitored by following the binding of TM to actin in function of myosin S1 concentration using direct co-sedimentation assay. S1 was able to induce cooperative actin binding of the mutants, that was manifested by sigmoidal shape of the binding curves. Thus, removal of the direct contact between tropomyosins did not eliminate cooperativity of TM binding in the "open" state. C-terminal truncations of both TM isoforms significantly increased the number of myosin heads required for saturation of the actin filament with tropomyosin and the effect was larger for the isoform with the end encoded by constitutive exon 9d. Moreover, although the N-terminal regions of both isoforms were identical, removal of nine N-terminal amino acids had stronger effect on TM9d than on TM9a. Subsequent removal of the C-, N- and both termini from TM9a was additive, whereas the effect of N-terminal truncation of TM9d was similar to double truncation. This suggests that tropomyosin with the C-terminus 9d requires N-terminal sequences of the following tropomyosin molecule for proper functions. These experiments indicate that forming a continuous cable along the actin filament is not necessary for maintaining cooperativity of TM binding in the "open" state. Most probably the main function for tropomyosin's ends is to confer high actin affinity. Since truncation lowered tropomyosin affinity without eliminating cooperativity with myosin S1, the source of the cooperativity must be a conformational change in actin that occurs upon S1 binding and is propagated to the neighboring actin molecules. This manifests itself by cooperative binding of tropomyosin. This idea is supported by the observation that binding of truncated tropomyosins to actin fully decorated with myosin heads was non-cooperative. Once myosin changed actin structure, tropomyosin bound tightly and non-cooperatively (Moraczewska & Hitchcock-DeGregori, 2000).

More information on the contribution of the ends to the cooperativity of the closed-to-open transformation comes from a study in which different tropomyosins with alternative N-termini encoded by exons 1a or 1b, in combination with alternative C-termini encoded by exons 9a, 9c or 9d, were compared in a myosin-induced tropomyosin binding assay (Moraczewska *et al.*, 1999). The cooperativity with which myosin heads induced binding of tropomyosin to actin correlated with the actin affinity of TM variants. The higher the affinity, the lower was the number of strongly bound myosin heads required to saturate actin with TM.

This rule does not apply, however, when the contribution of tropomyosin's inner actin-binding repeats to the cooperativity of activation is considered. Analysis of αTM mutants with progressively larger deletions of internal periodic repeats revealed that TM affinity for actin in the "blocked" or "closed" state (with Tn ± Ca^{2+}) does not correlate with tropomyosin binding in the "open" state. Cooperativity of the closed-to-open transition of deletion mutants lacking two (periods 2 and 3) or three periods (2–4) was not related to actin affinity of the mutant in the absence of myo-

sin. The cooperativity considered in terms of TM length, which for the three-period deletion mutant was only about 60% the length of native TM, was unchanged. Both mutants required the same number of myosin heads per TM molecule as wild-type TM to saturate the actin filament (Hitchcock-DeGregori *et al.*, 2001). Interestingly, when period 5 was included in the deletion (deletion of periods 3-5), myosin-S1 was not able to induce binding of tropomysin in the "open" state (Landis *et al.*, 1999; Hitchcock-DeGregori, 2001). This indicates that not the length of TM, but rather specific regions of TM determine interactions with S1.

Examining the actin binding properties of other TM mutants with two or three actin periods deletions, Tobacman and colleagues came to the conclusion that actin binding periods 3 to 5 greatly contribute to the energetics of TM binding in the "open" state (Landis *et al.*, 1999). Deletions of inner TM regions affect myosin binding to actin in a similar way: they affect binding of the same tropomyosin mutants to actin saturated with myosin. The mutants in which periods 3, 4 or 5 are deleted strongly inhibit actin binding of S1-ADP at low S1 concentrations and cause a sharp increase in the affinity for actin at higher S1 concentrations (Tobacman & Butters, 2000; Rosol *et al.*, 2000). These findings suggest that the quasi-repeating TM regions are not equivalent in the interaction with myosin, despite the fact that TM molecules form a continuous cable along the filament.

Taking into account that actin and TM play an active role in the thin filament activation, Tobacman & Butters (2000) proposed a model of cooperative myosin-thin filament binding. The model assumes that the same conformational change in actin, which is induced by myosin binding, is promoted by TM interacting with actin inner domain in the "open" state. Myosin induces the conformational change in actin and facilitates repositioning of tropomyosin on the filament. Once tropomyosin is shifted from its "closed" to the "open" state position, it promotes tight myosin binding. This concerted action of myosin and tropomyosin in actin transition into the active state produces cooperative effects. Various tropomyosin regions participate unequally in this process. The greatest contribution to the equilibrium constant for the conformational change in actin comes from central regions of tropomyosin. If the inner segment has to be shifted to promote the myosin-induced change in actin, then the deletion mutant depleted of internal regions is not moved easily from the position and it sterically blocks the myosin binding sites on actin. This implies that TM flexibility is a factor of great importance in the cooperative activation process.

The structural basis for TM flexibility is given by the recent atomic structure of a recombinant 81-residue N-terminal peptide of chicken striated αTM. The structure revealed that the interface between α-helices consists of alternating segments. Segments rich in alanine intertwine with segments containing branched hydrophobic residues like leucine. The parallel helices in the alanine segment are axially shifted, whereas in the leucine segments the helices remain in-register. This generates bands that are a source of significant conformational variety of the molecule (Brown *et al.*, 2001).

SUMMARY

It is not the end-to-end overlap between neighboring tropomyosins that is required for cooperativity to occur. The role of alternative ends of TM is rather to confer actin affinity specific for TM each isoform. Tropomyosin affinity to actin is related to the size of the cooperative unit; the higher the affinity, the smaller the number of myosin heads required for full activation. TM inner sequences, particularly the sequences comprising actin-binding periods 3 and 5, are crucial for myosin-induced activation of the thin filament. Since

the tropomyosin molecule has a potential for conformational variety, it is plausible that its inner regions adopt the right conformation facilitating actin conformational change. An increasing number of data supports the involvement of actin conformational change in cooperative transitions within the thin filament, however, to explain the exact nature of these changes the structure of the thin filament at atomic resolution has to be solved.

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