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Phosphorylation of Ser283 enhances the stiffness of the tropomyosin head-to-tail overlap domain

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ABSTRACT

The ends of coiled-coil tropomyosin molecules are joined together by nine to ten residue-long head-to-tail "overlapping domains". These short four-chained interconnections ensure formation of continuous tropomyosin cables that wrap around actin filaments. Molecular Dynamics simulations indicate that the curvature and bending flexibility at the overlap is 10–20% greater than over the rest of the molecule, which might affect head-to-tail filament assembly on F-actin. Since the penultimate residue of striated muscle tropomyosin, Ser283, is a natural target of phosphorylating enzymes, we have assessed here if phosphorylation adjusts the mechanical properties of the tropomyosin overlap domain. MD simulations show that phosphorylation straightens the overlap to match the curvature of the remainder of tropomyosin while stiffening it to equal or exceed the rigidity of canonical coiled-coil regions. Corresponding EM data on phosphomimetic tropomyosin S283D corroborate these findings. The phosphorylation-induced change in mechanical properties of tropomyosin likely results from electrostatic interactions between C-terminal phosphoSer283 and N-terminal Lys12 in the four-chain overlap bundle, while promoting stronger interactions among surrounding residues and thus facilitating tropomyosin cable assembly. The stiffening effect of D283-tropomyosin noted correlates with previously observed enhanced actin–tropomyosin activation of myosin S1-ATPase, suggesting a role for the tropomyosin phosphorylation in potentiating muscle contraction.

Introduction

Post-translational phosphorylation and modification of cardiac muscle proteins are hallmarks of cardiovascular responses and adjustments to physiological challenges [1,2]. Sarcomeric proteins targeted for phosphorylation include cardiac myosin light chains (MLC-2)[4], myosin binding protein C (MyBP-C), troponin I (TnI), troponin T (TnT), tropomyosin and titin [1–12]. However, the interplay between the effects of post-translational modification remains unclear. In fact, unlike the phosphorylation of most proteins, tropomyosin phosphorylation, studied here, is not subject to minute-to-minute change [7], suggesting that the modulation is a comparatively long-term functional adaptation. Tropomyosin phosphorylation in striated muscle appears to be restricted to serine 283, the penultimate residue in the 284 amino acid long tropomyosin sequence [7,8]. S283 phosphorylation is found to be highest in fetal hearts (~70% phosphorylated) but declines slowly post-partum and with increasing age (to ~20–30%), suggesting a developmental role possibly related to myofilament assembly [13]. In some cases, a variable increase in phosphorylation is observed in diseased adult hearts [14], correlated with possible protein expression regressing to that of the fetus.

The regulation of cardiac muscle actin–myosin interaction and hence contractile force is governed by the thin filament regulatory proteins tropomyosin and troponin and their interactions with actin, myosin and Ca^{2+} [15,16]. 40 nm long tropomyosin coiled-coils

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[4] *Abbreviations used:* MLC-2, cardiac myosin light chains; MyBP-C, myosin binding protein C; TnI, troponin I; TnT, troponin T.

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associate head-to-tail on thin filaments to form a continuous cable that follows the helical path defined by actin subunit interactions. Each molecule of tropomyosin spans seven actin monomers and binds to one troponin complex containing troponin subunits TnT, TnI and TnC. Under the control of Ca^{2+}-binding to troponin and myosin binding to actin, tropomyosin translocates azimuthally across the actin filament, to transiently block or open myosin-binding sites on actin, thereby regulating myosin-head cross-bridge cycling on actin and consequently contractility [17–19].

Single tropomyosin molecules bind to actin filaments with exceedingly low affinity. Tropomyosin only binds to actin filaments with appreciable affinity after head-to-tail polymerized molecules populate actin filaments and begin to form a cable [20]. Thus, effective binding results from the collective interactions of tropomyosin molecules in the cable and the successive actin subunits along thin filaments [18]. Since the tropomyosin molecule is semi-rigid, relatively long stretches of the cable, greater than the 40 nm length of single tropomyosin molecules, can move azimuthally as a unit over actin, implying that the head-to-tail connection also is semi-rigid [21]. Thus once assembled, the actin–tropomyosin co-filament cooperatively activates myosin ATPase and cross-bridge cycling.

Tropomyosin is a canonical coiled-coil along most of its length [21–23]. However, the two-chained coiled-coil architecture is disrupted over C-terminal 20–25 residues during tropomyosin polymerization [24–26]. Here tropomyosin splays apart to accommodate a more compact N-terminal end of an adjacent molecule [24–27]. Thus, the resulting four-coiled bundle/overlap domain is the "glue" responsible for tropomyosin polymerization into a cable. Phosphorylation of serine 283, located at the center of the head-to-tail tropomyosin junctional complex, may therefore influence tropomyosin cable assembly and stability. In the current investigation, we examined this possibility by studying the structural and functional effects of striated muscle α-tropomyosin (i.e. Tpm1.1st (a.b.b.a) [28]) phosphorylation in order to determine whether or not phosphoserine-283 affects the mechanical and chemical properties of the tropomyosin overlap domain. MD simulation and EM data acquired indicate that Ser283 phosphorylation straightens the tropomyosin overlap domain to match the curvature of the remainder of tropomyosin and to fit better to the actin–filament helix. Moreover, the phosphorylation stiffens the overlapping domain to equal or exceed the rigidity of canonical coiled-coil regions of tropomyosin. The conformational adjustment may potentiate tropomyosin cable translation across the actin filament and account, at least in part, for the phosphorylation-induced enhanced acto-S1 activation by actin–tropomyosin [29,30].

Materials and methods

Reference models for MD simulations

The initial reference models used for MD of the striated muscle α-tropomyosin (Tpm1.1st (a.b.b.a)) head-to-tail overlap domain segment were based on a corresponding NMR structure (PDB ID code 2g9j:conformer model-1) [26]. In the work reported here and in Ref. [31], we replaced the peripheral non-tropomyosin residues used to stabilize overlap domain peptides examined in the NMR study with native coiled-coil sequences to build an 80-residue-long fragment of the tropomyosin polymer consisting of 40 residue contributions from the C- and N-termini connected by the 10-residue long head-to-tail nexus. We used native acetylated N-terminal methionine residues [*cf.* 31–33] instead of the amino acid mimetics shown in the PDB structure [26]. The initial reference model containing phosphorylated serine 283 was then built from the average MD structure of the domain [31] and the serine hydroxyl side-chain replaced with phosphate using the CHARMM SP2 patch [34]. Thus, the initial reference models built in the current study and used for MD simulations here are exactly the same as those in our previous work [31], except for the substitution of phosphoserine for serine at residue 283 of tropomyosin. Control studies examining the effect of phosphoserine 283 on isolated 40-residue long "non-polymerized" C-terminal fragments of tropomyosin were initiated from reference models cut from full length tropomyosin [21].

Molecular Dynamics and analysis

The reference models were energy minimized [21,31,35] and Molecular Dynamics simulations performed in explicit solvent including 150 mM NaCl at 310 °K, using NAMD version 2.9 [36] and the CHARMM27 force field [34,37,38] as previously described [21,31,35]. Analysis was carried out after discarding the first 4–5 ns of MD, ensuring that the variance of the measurements had stabilized [21,31,35]. MD trajectories were averaged as previously after discarding the first 4–5 ns in each simulation; averages were composed of snapshots sampled every picosecond during the trajectory [21,31,35]. The curvature of the tropomyosin overlap domain was quantified by determining the angle between the respective central axes of the C- and N-terminal coiled-coils fragments as diagramed in Fig. 2 of Ref. [31]. Persistence length was calculated by applying the tangent correlation method on snapshots taken from the MD trajectory as described previously [21] and in detail in [22]. The program Chimera [39] was used to display the MD averages.

Protein preparation

Standard methods were used to purify actin and to prepare F-actin and myosin subfragment 1 (S1) [40,41].

Preparation of recombinant wild-type and mutant tropomyosin

The cDNA of α-tropomyosin (Tpm1.1st (a.b.b.a)) was obtained using mouse heart total RNA as a template and following standard protocols of the Fermentas First Strand cDNA Syntheses kit (Thermo Scientific, Pittsburgh, PA). The cloning of the cDNA was further carried out using a TA PCR cloning kit (Invitrogen-Life Technologies, Grand Island, NY). Nine extra nucleotides (ATGGCTAGC) translating into Met-Ala-Ser were added at N-termini of the cDNA to mimic acetylation required for head-to-tail tropomyosin assembly [42]. Site directed mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to replace serine 283 in tropomyosin with either aspartic acid to mimic the phosphorylated state (S283D) or alanine as a control (S283A). A pET-24 (Novagen, Madison, Wisc.) vector containing the T7 promoter, lac operator and a kanamycin resistant gene was used for the expression of wild-type and mutant proteins in *Escherichia coli* (BL21). The DNA sequences of the expression constructs were verified by DNA sequencing and confirmed the presence of Ala-Ser at the tropomyosin N-terminus. The expressed tropomyosin was extracted from bacterial cells by sonication in 25 mM Tris buffer (pH 8), 25 mM NaCl, 2 mM EDTA, and 0.1% Triton X-100. The protein was then purified by three cycles of precipitation at pH 4.6 and resuspension in 1 M KCl buffer (pH 7), followed by ammonium sulfate fractionation. The protein precipitated between 65% and 70% $(NH_4)_2SO_4$ saturation was collected and dialyzed against 2 mM β-mercaptoethanol and then lyophilized. The purity of the protein was assessed by SDS PAGE.

Electron microscopy and persistence length determination

Samples of control (wild-type), S283D and S283A tropomyosin constructs were rotary shadowed and electron microscopy carried