thin filaments, which contain only one troponin for every tropomyosin, it is the middle, fourth quasi-repeat of tropomyosin which interacts with TnI C-terminus.

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Surface Immobilization of Cardiac Thin Filaments

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Our objective is to directly observe a single troponin (Tn) complex exchange among its conformational substates. Here, we report experimental conditions for immobilizing thin filaments (TF) to glass coverslips. TF were reconstituted from F-actin, tropomyosin (cTm), and cTn, and were sonicated to produce defined lengths. PEG-functionalized, neutravidin-coated coverslips were prepared, and biotinylated gelsolin was sparsely immobilized to neutravidin. TF were captured from solution in a flow chamber, and imaged under oxygen scavenger solution. Labeled TF cTn(TnC-C89*AF488) (blue), cTm-C190*AF546 (green), phalloidin*AF647 (red) were assessed by three-color widefield fluorescence. Colocalization of blue, green, and red confirmed that thin filaments contained Tn, Tm and actin. The number of dye-labeled cTn per TF was quantified by the number of photo bleaching events per thin filament. We used photobleaching to confirm our ability to prepare TF with one or fewer dye-labeled cTn per filament. These results demonstrate our ability to reconstitute fluorescent-labeled thin filaments immobilized in PEG-coated flow chambers.

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Structural and Kinetic Studies using FRET: Impact of Pseudo-Pkc Phosphorylation of Cardiac Troponin T on Calcium-Activated Thin Filament Regulation

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Regulation of myocardial contractility is influenced by numerous signaling agents. One of the key methods for modification of cardiomyocyte function is phosphorylation of cardiac troponin (cTn), the Ca^{2+}-dependent regulatory switch that controls thin filament activation, by isoforms of protein kinase C (PKC). PKC has been shown to phosphorylate the tropomyosin binding subunit of cTn (cTnT) at Thr-195, Thr-199, Thr-204 and Thr-285 (rat cTnT). The effect of these phosphorylations on cTn structure and dynamics remains largely unknown. To ascertain the site specific effects of PKC phosphorylation of cTnT on thin filament regulation, pseudo-phosphorylation of cTnT was combined with Förster resonance energy transfer (FRET) to monitor the transition between the Ca^{2+} and Mg^{2+} states of cTn. Single-cysteine cTnI mutants were labeled with IAEDENS at either Cys-151 or Cys-167, and a single-cysteine cTnC mutant was labeled at Cys-89 with DDPM. Different combinations of mutant cTnT and fluorophore labeled cTnI and cTnC were then incorporated into reconstituted thin filaments, which were subjected to calcium titrations, time resolved FRET structural measurements, and stopped flow kinetic measurements. Titrations showed a consistent pseudo-phosphorylation dependent increase in pCa50 values, and kinetics experiments showed decreased rates of Ca^{2+} dissociation in pseudo-phosphorylated mutants. Additionally, Mg^{2+} distances between Cys-167 of cTnI and Cys-89 of cTnC were reduced in the presence of pseudo-phosphorylation. Thus our results suggested that pseudo-phosphorylation of cTnT leads to sensitization of the Ca^{2+}-dependent conformational transitions involved in cTn regulatory switching. However, a prior in situ study demonstrated a pseudo-phosphorylation dependent decrease in EC50 (JBC vol. 278, pp. 35135-44). These results may suggest PKC mediated phosphorylation of cTnT may represent a post-translational modification where cTn regulatory switching is sensitized to Ca^{2+}, but also partially uncoupled from Tm movement.

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Structural and Kinetic Effects of HCM Related Mutations R146G/Q and R163W of Cardiac Troponin I on cTnI-cTnC Interaction within Reconstituted Thin Filament

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Mutations in cardiac troponin I (cTnI) that cause hypertrophic cardiomyopathy (HCM) have been reported to change the contractility of cardiac myofilaments, but the underlying molecular mechanism remains elusive. In this study, fluorescence spectroscopy was used to investigate the specific structural and kinetic effects that HCM related cTnI mutations R146G/Q and R163W exert on Ca^{2+} and myosin S1 dependent conformational transitions in cTn structure while in the reconstituted thin filament. Fluorescently labeled mutants of cTnC and cTnI were prepared for this purpose. Ca^{2+}-induced changes in interactions between cTnC and the inhibitory region ($cTnI_{IR}$) and regulatory region ($cTnI_{RR}$) of cTnI were individually monitored using steady-state and time-resolved Förster resonance energy transfer, and kinetics were determined using FRET stopped-flow. It was found that R146G/Q and R163W all changed the molecular distances between cTnC and $cTnI_{IR}$ and $cTnI_{RR}$ in unique and various ways. However, kinetic rates of conformational transitions induced by Ca^{2+}-dissociation were universally slowed when R146G/Q and R163W were present, except that R146Q sped up changes in the $cTnI_{RR}$–cTnC interaction. This explained the universal trend of increased Ca^{2+}-sensitivity. Interestingly, the kinetic rates of changes in the $cTnI_{IR}$–cTnC interaction were always slower than that of the $cTnI_{RR}$–cTnC interaction, suggesting that the flycasting mechanism that normally underlies deactivation is preserved in spite of mutation. Thus our results revealed that R146G/Q and R163W each impact regulatory switching in their own unique way, but with a common kinetic outcome: the slowing of thin filament deactivation. Furthermore, R146Q and R163W disrupted the blocked state, causing the thin filament to behave as if it is in the fourth "pre-relax" state of regulation.

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Long-Range Transmission of Structural Instability due to the FHC Mutation E180G in Coiled-Coil Tm is inhibited by an Interchain Disulfide Bond

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Previously we showed that the familial hypertrophic cardiomyopathy (FHC) mutation in Tm, E180G, increased the trypsin cleavage rate at R133 and introduced another cleavage site with a similar rate at K233 (Ly & Lehrer, 2012). To understand how this mutation can produce this dynamically unstable hot spot 53 residues away, we introduced a disulfide crosslink (XL) at C190 and studied its effect on limited trypsin digestion using MALDI MS and N-terminal sequencing.

Trypsin digestions at 26° of native disulfide crosslinked (XL) ααTm, FHC mutant D175N and WT, produced a XL heterodimer intermediate for all, containing one cleaved chain at R133, XL to the full length chain (134-284/1-284) (50 kDa) as well as the doubly cleaved XL homodimer (134-284/134-284) (35 kDa). For XL E180G, however, another intermediate was also present (169-284/1-284) (46 kDa). For native XL ααTm, the cleavage site at K168 was only observed at 40° where the middle of Tm is unfolded. In contrast to uncrosslinked E180G, where cleavage occurred at residue 233, no cleavage was observed at residue 233 for crosslinked E180G.

These results show: 1) that in addition to the increased destabilization near R133 due to the Glu to Gly change at residue 180 in XL E180G, further destabilization occurs in the region near residue 168; 2) the long-range destabilization near residue K233 caused by the E180G FHC mutation is inhibited by the intervening XL. This suggests that interchain dynamics is involved in propagating the local perturbation at 180G due to the mutation to the K233 region.

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Instability of D145E, a Cardiac Troponin C Mutant Linked to Cardiomyopathy, Dictates Profound Changes in Ca^{2+} Regulation of Myofibrils at Increased Temperature

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We have shown previously that the hypertrophic cardiomyopathy mutant HcTnC D145E lacks α-helical content compared to WT and confers greater Ca^{2+} sensitivity on regulated thin filaments at 21°C. We hypothesize that the functional alterations are caused by protein instability. Here, using spectroscopy and skinned-fiber assays, we examined the thermodynamic and functional parameters of HcTnC D145E at physiologically relevant temperatures. Based on circular dichroism, isolated D145E lost more of its secondary structure than did WT at high temperatures (30-45°C). Melting temperatures Tm for WT and D145E were 71.8 ± 0.2 vs 51.5 ± 0.8°C in Mg^{2+} (1 mM free)+ EGTA and 78.2 ± 0.2 vs 55.7 ± 1.0°C in 1 mM free Mg^{2+}, pCa 4. In 3M urea (with and without Ca^{2+}/Mg^{2+}), D145E lost nearly all secondary structure at 10°C while WT began to unfold only at 45-50°C. using bis-ANS, a fluorescent probe that binds to hydrophobic surfaces, we found that apoD145E was structurally more open than WT, while Ca^{2+}, which opens hydrophobic pockets in apoTnCWT, had no additional effect on D145E. The Ca^{2+} affinity increment (ΔpCa50) (=pCa50D145E-pCa50WT) of isolated D145E vs WT