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Mutagenesis of a Tropomyosin Isoform from Atlantic Salmon
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Atlantic salmon striated muscle tropomyosins unfold over a lower temperature range than their mammalian counterparts. A single sarcomeric isoform belonging to the alpha-class exists in the fast skeletal muscle of the trunk. It shares 20 amino acids substitutions with rabbit alpha-skeletal tropomyosin but migrates closer to the beta isoform (40 substitutions) when electrophoresed in the presence of SDS. Interesting aspects of the sequence heterogeneity (versus rabbit alpha) include: a change in charge at neutrality - salmon fast skeletal tropomyosin is of greater net negative charge - the content of core residues in the carboxyl-terminal half of the molecule and glycine in the amino-terminal half. Mutation of threonine-77 in salmon to the corresponding residue in rabbit, lysine, results in an electrophoretic shift in the absence as well as the presence of SDS. The contribution of the various isomorphisms to the properties of salmon tropomyosin is under investigation using circular dichroism and limited proteolysis.

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Structural Instability of Tropomyosin FHC Mutants D175N and E180G Probed by Limited Trypsin Cleavage
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Physiological and biochemical studies have shown that intact and reconstituted muscle thin filaments containing FHC mutants of tropomyosin Tm-D175N and Tm-E180G have a greater Ca2+-sensitivity than native systems. These mutant molecules have a lower thermal stability and bind less strongly to actin (Golitsina et al., 1997; Kremneva et al., 2004). Here we report trypsin digestion kinetics and cleavage sites using PAGE-SDS gels, MALDI MS and N-terminal sequencing. Native Tm is initially cleaved at Arg 133 to form a C-terminal 18KDa and an N-terminal 15KDa fragment due to the instability caused by a nearby Asp 137 in the hydrophobic ridge of the coiled-coil α-helix (Pato et al., 1981; Sumida et al., 2008). WT, Tm-D175N and Tm-E180G were also cleaved at R133, but Tm-D175N and Tm-E180G were cleaved 1.3X and 3.3X faster than WT, respectively. Cleavage of Tm bond to actin was slowed an order of magnitude with similar order of rate: E180G > D175N > WT.
In the absence of actin, Tm-E180G, is cleaved at K233 (as well as R133), to produce intermediates of 27KDa and 6KDa. At longer times, the 18KDa fragment of all mutants gets cleaved at R167 to produce a 13.5KDa fragment. For Tm-E180G, the N-terminal 27KDa intermediate is similarly cut at R167 to produce the15KDa fragment and a new 12KDa fragment. ATPase studies of reconstituted thin filaments showed an elevated ATPase at low Ca2+ with: Tm-E180G > Tm-D175N > WT. Thus, whereas both Tm-D175N and Tm-E180G show greater instability near R133 than WT, for Tm-E180G there is an additional region of dynamic instability, near K233. The increased instability for both mutants would result in greater flexibility which appears to be involved in the increased Ca2+-sensitivity observed. Supported by NIH HL 91162.

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Geometry of the C-/N-Terminal Connection of Tropomyosin on F-Actin Based on Molecular Dynamics Simulations of the Thin Filament
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The binding of tropomyosin molecules to actin filaments is extremely weak and only is effective because of cooperative interactions resulting from the head-to-tail polymerization of tropomyosin on thin filaments. Experiments show that interfering with the head/tail interactions prevents actin-binding (Johnson and Smillie, 1977; Heald and Hitchcock-DeGregori, 1988). Crystal- and NMR-structures of mixtures of head/tail-fragments stabilized with various foreign adducts, yet unconstrained by interactions with F-actin, have suggested that head-to-tail linkage results from a splayed C-terminal coiled-coil region enveloping a more compact N-terminus to form a four-helix nexus with varying degree of overlap. However, it is possible that structures observed previously may have been influenced by the adducts and the absence of F-actin. In fact, N-/C-terminal sequences vary greatly between tropomyosin isoforms and are not strictly tetra-coiled-coiled sequences, complicating interpretation. Here, based on static and dynamic models of $\alpha\alpha$-striated muscle tropomyosin on F-actin, we derive the overlap and the relative pseudo-rotation angle (which determines the face of tropomyosin which is seen by F-actin) between the C- and N-terminal ends. Our recently described atomic model of tropomyosin on F-actin (Li et al., 2011) suggests that, when constrained by interaction with the F-actin surface, the tropomyosin ends overlap by five amino acids, slightly shorter than in the adduct-structures. MD simulations provide a measure of the variability in end-to-end distance of tropomyosin on F-actin (and therefore of the possible overlap length) and in the degree of pseudo-rotation between N- and C-tropomyosin termini (and therefore C-/N-nexus geometry). These simulations demonstrate local C-terminal coiled-coil splaying, relatively short overlap (7.9 $\pm$ 2.2Å, i.e. ~four to seven residues) and predict a rotation of 17.5 $\pm$ 13° at the end of one tropomyosin relative to the beginning of the next.

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The Functional Consequences of HCM Troponin C Mutations in the Regulation of Slow Skeletal Muscle Contraction
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The cardiac troponin C (cTnC) subunit of the troponin complex is expressed in both slow skeletal and cardiac muscle. Therefore, investigating the effects of cTnC mutations associated with hypertrophic cardiomyopathy (HCM) in slow skeletal muscle may reveal new insights into the mechanisms underlying cardiomyopathies. Glycerol SDS-PAGE analysis was performed on rabbit soleus muscle in order to determine the amount of slow myosin heavy chain type I (MHC I) present and its ratio to other myosin isotypes. The results show that the entire rabbit soleus muscle consists of MHC-1 slow type fibers; thereby, making it possible to use rabbit soleus muscle as a model system for slow skeletal muscle experiments. In order to determine the effects of TnC mutations on the Ca^{2+} sensitivity of contraction in skinned slow muscle fibers, native TnC was extracted and reconstituted with WT or mutant TnCs. The A8V, E134D, D145E and C84Y cTnC mutants were all tested and C84Y-cTnC was the only mutant that increased the Ca^{2+} sensitivity of force development in both the skinned cardiac and slow skeletal fibers. Additionally, none of the mutants affected the restored maximal force in the soleus fibers. The absence of a phenotype arising from most of the mutants in the soleus muscle suggests that the aberrant effects of cardiomyopathic cTnCs may be tissue specific, such that other proteins present in slow skeletal muscle may potentially rescue the deleterious effects of the mutations. In the next set of experiments we will determine if these mutations alter the energetics of crossbridges by evaluating the Actomyosin ATPase activities of soleus myofibrils that have their native TnC extracted and reconstituted with recombinant mutant cTnCs. Supported by J&E King 1KD03-33923 (DD), NIH R01-HL42325 (JDP) and NIH 1K99HL103840-01 (JRP).

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The Influence of Troponin C, Isoform 4 on Drosophila Development, Stretch Activation, and Power Generation
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Drosophila indirect flight muscle (IFM) is one of the most rapidly contracting muscle types known. To increase IFM efficiently and power generation many insect species have evolved high degrees of stretch activation and shortening deactivation in their IFMs. In addition to expressing TnC1, a typical calcium binding troponin C, these IFMs express an unusual unique, additional isoform, TnC4. TnC1 has two calcium binding sites, a high-affinity structural site and a lower-affinity regulatory site. TnC4 has only the high-affinity structural binding site. It is hypothesized to respond to stretch rather than calcium concentration to further activate the thin filament. We are investigating the roles of TnC4 and TnC1 in *Drosophila* using RNAi and by creating a TnC4 null mutant. Using RNAi, we eliminated TnC1 expression, which resulted in up-regulation of TnC4. Power generation and stretch activation of IFMs without TnC1 were statistically identical to wild-type. IFMs with undetectable levels of TnC4 expression produced by RNAi do not generate power or display stretch-activation, suggesting that only TnC4 is necessary for normal muscle function. We have also created a TnC4 null, which will allow us to mechanically evaluate transgenically expressed TnC isoforms and mutants in IFM. Preliminary characterization of the TnC4 null indicates that it is homozygous lethal, but viable as a heterozygote. Flight ability of the heterozygotes is reduced, displaying a 10% reduction in wing-beat frequency than wild-type. The homozygous nulls die at the first molt suggesting that TnC4 is also expressed in some larval muscle types and that it plays a vital role in fly development.