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Troponin Regulatory Function and Dynamics Revealed by H/D Exchange-Mass Spectrometry

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Troponin is the thin filament protein that confers tight, Ca^{2+}-dependent control over muscle contraction. The mechanism of this regulation was investigated by detailed mapping of the dynamic properties of cardiac troponin, using amide hydrogen exchange-mass spectrometry, in the presence of either saturation or non-saturation of the regulatory Ca^{2+} binding site in the NH_2-domain of subunit TnC. Troponin was found to be highly dynamic, with 60% of amides exchanging H for D within seconds of exposure to D_2O. In contrast, portions of the TnT-TnI coiled-coil exhibited high protection from exchange, more than six hours, identifying the most stable portion of the trimeric troponin complex. Regulatory site Ca^{2+} binding altered dynamic properties (i.e., H/D exchange protection) locally, near the binding site and in the TnI switch helix that attaches to the Ca^{2+}-saturated TnC NH_2-domain. More notably, Ca^{2+} also altered the dynamic properties of other parts of troponin: the TnI inhibitory peptide region that binds to actin, the TnT-TnI coiled-coil, and the TnC COOH-domain that contains the regulatory Ca^{2+} sites in many invertebrate as opposed to vertebrate troponins. Mapping of these affected regions onto troponin's highly extended structure indicates contacts important in conformational change: in the low Ca^{2+} state the TnI region that effects inhibition bends back and interacts with the end of the TnT-TnI coiled-coil, as previously suggested by intermediate resolution X-ray data of skeletal muscle troponin. Thus, troponin-mediated Ca^{2+} sensitive regulation of muscle contraction consists of Ca^{2+}-triggered switching between alternative sets of intra-troponin interactions.

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Magnesium Stabilizes the Closed Conformation of the C-Domain of Troponin C

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Activation of the thin filament in striated muscles requires both the binding of Ca^{2+} to the N-domain of troponin C (TnC) and the binding of myosin cross-bridges to actin, which has been shown to alter the C-domain conformation. Here we have evaluated the structural and functional consequences of divalent cation exchange in skeletal and cardiac TnC (sTnC and cTnC). We have used intrinsic tyrosine fluorescence, circular dichrosim (CD), and the fluorescent nonspecific hydrophobic probe bis-ANS to monitor changes in domain conformation in response to Ca^{2+} and Mg^{2+} binding in the sTnC, cTnC, and in a cTnC mutant in which the invariant Glu residue at the 12th position of the calcium binding loops III and IV were substituted with Asp (cTnCDD). Ca^{2+} binding causes an increase in Tyr fluorescence and α-helical content in sTnC and cTnC, but not in cTnCDD. Ca^{2+} induced C-domain opening characteristic of sTnC and cTnC was also greatly reduced in cTnCDD, as measured by bis-ANS fluorescence. Thus the Asp to Glu substitutions appear to prevent the C-domain from opening. Bis-ANS Ca^{2+} titrations also showed that high Ca^{2+} concentrations may be sufficient to open the N-domain of cTnC, which was reported to remain in the closed conformation in the Ca^{2+}-bound state. Lastly, bis-ANS Mg^{2+} titrations indicate that Mg^{2+} does not cause domain opening in either cTnC or cTnCDD. The closed conformation of the Mg^{2+}-bound C-domain of TnC implies a different mechanism of interaction with TnI than that in the presence of Ca^{2+} and suggests that the Mg^{2+}-Ca^{2+}exchange in TnC may contribute to the thin filament activation of muscle contraction. This conclusion is consistent with our observation that physiological concentrations of Mg^{2+} significantly lower the Ca^{2+}-sensitivity of reconstituted cardiac thin filaments.

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Phosphomimetic Substitutions in One or Both Ser43/45 Residues of Cardiac Troponin I Produces Comparable Changes in Contractile Performance

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Cardiac troponin I (cTnI) is phosphorylated on three clusters of residues in response to protein kinase C (PKC) activation. Previously, studies on the cTnISer43/45 cluster showed phosphomimetic Asp substitution reduced peak shortening and accelerated re-lengthening in adult cardiac myocytes. The goal of the present study is to determine whether one or both Ser residues contribute to the functional response observed with cTnISer43/45Asp. We studied adult rat cardiac myocytes 2 and 4 days after viral-mediated gene transfer of cTnIFLAG, cTnISer43Asp or cTnISer45Asp (+FLAG). Western analysis indicated similar levels of cTnI replacement developed for all groups, and extensive replacement with cTnIFLAG ($71 \pm 9\%$, n= 6), and FLAG-tagged epitopes of cTnIS43D ($72 \pm 3\%$, n=8) and cTnIS45D ($70 \pm 5\%$, n=8) within 4 days. Further analysis showed no significant change in cTnI stoichiometry and confocal analysis confirmed a sarcomeric incorporation pattern for each mutant. In functional studies, shortening amplitude decreased significantly in chronically paced myocytes expressing non-tagged Ser43Asp and/or Ser45Asp compared to controls (Control = 0.149 ± 0.008 μm, n=36; cTnISer43/45Asp = 0.110 ± 0.006 μm; n=32*; cTnISer43Asp = 0.095 ± 0.007, n=44*; cTnISer45-Asp = 0.108 ± 0.007*, n=50; *$p<0.05$ vs control) 4 days after gene transfer. An accelerated re-lengthening accompanied this reduced shortening (Time to 75% relaxation = $TTR_{75\%}$ (ms): Control = 79 ± 4; cTnISer43/45Asp = 62 ± 4*; cTnISer43Asp = 63 ± 4*, cTnISer45Asp = 65 ± 3*; *$p<0.05$ vs control). Interestingly, each single mutant also accelerated the time to peak shortening (TTP (ms): Control = 83 ± 5; cTnISer43Asp = 68 ± 3*; cTnISer45Asp = 67 ± 2*; *$p<0.05$ vs control) while cTnISer43/45Asp did not (84 ± 5). These initial results provide evidence that each Ser residue in the Ser43/45 cluster is capable of altering cTnI function in response to phosphorylation by PKC, yet phosphorylation of both residues does not produce an additive response.

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Effect of Troponin Ca^{2+} Binding Properties on the Kinetics of Myofibril Force Initiation and Relaxation

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We have engineered the Ca^{2+} binding properties of troponin C (TnC) to study the role of increased (I60Q sTnC) and decreased (M80Q $sTnC^{F27W}$) Ca^{2+} dissociation rate (k_{off}) on activation and relaxation of skeletal muscle. Previously we reported that myofibril force development kinetics (k_{ACT}) are not influenced by decreasing k_{off} from Tn, but are slowed by an increase in k_{off} (Kreutziger et al. 2008 JPhsiol. 586;3683-3700) at low [P_i] (5 μm). The time to initiation of force ($k_A$lag) following a rapid (~10ms) switch from pCa 9.0 to pCa 3.5 provides information about thin filament activation rate and our preliminary data suggest this rate may also be sensitive to k_{off}. In rabbit psoas myofibrils (15°C) $k_A$lag (~20 ms for native or WT sTnC) is almost eliminated for M80Q $sTnC^{F27W}$ and increased by I60Q sTnC (~40-50 ms). Additionally, though k_{ACT} is similar for force increases from either full or partial activation to full activation, $k_A$lag disappears when starting from partial activation. We have also reported that fast and slow phase rates of relaxation are not affected by k_{off}, but that duration of the slow phase is affected in skeletal myofibrils. Here we report that lag prior to initiation of the slow phase ($k_R$lag) may be also influenced by k_{off}. Opposite to $k_A$lag, $k_R$lag (~20 ms for WT or native sTnC) was increased (~40-50 ms) by decreased k_{off} (M80Q $sTnC^{F27W}$) and almost eliminated by increased k_{off} (I60QsTnC). These experiments demonstrate a potential approach to study thin filament activation/deactivation kinetics without the need for fluorescent probes attached to thin filament proteins that can affect their function. Supported by Telethon GGP07133, MIUR (CP, CT), NIH-HL65497 (MR).

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Nebulin Alters Crossbridge Cycling Kineticis and Increases Thin Filament Activation - a Novel Mechanism for Increasing Tension and Reducing Tension Cost

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Nebulin is a giant filamentous F-actin binding protein that binds along the thin filament of the skeletal muscle sarcomere. Although nebulin is usually viewed as a structural protein, here we investigated whether nebulin plays a role in muscle contraction by using skinned muscle fiber bundles from a nebulin knockout (NEB KO) mouse model. We measured force-pCa and force-ATPase relations, as well as the rate of tension redevelopment (k_{tr}) in tibialis cranialis fibers. To rule out any alterations in troponin (Tn) isoform expression and/or status of Tn phosphorylation, we studied fibers that had been reconstituted with fast skeletal muscle recombinant Tn. We also performed a detailed analysis of myosin heavy chain, myosin light chain (MLC) and MLC2

phosphorylation, and found no significant differences between NEB KO and wt muscle. Our mechanical studies revealed that NEB KO fibers had increased tension cost (5.9 vs. 4.4 pmol mN^{-1} mm^{-1} s^{-1}) and reductions in k_{tr} (4.7 vs. 7.3 s^{-1}), calcium sensitivity (pCa_{50} 5.74 vs. 5.90), and cooperativity of activation (n_H 3.64 vs. 4.38). Our findings indicate that in skeletal muscle (1) nebulin increases thin-filament activation, and (2) that through altering crossbridge cycling kinetics, nebulin increases force and efficiency of contraction. In addition to nebulin deficient murine muscle, we also studied nebulin-deficient muscle fibers from patients with Nemaline Myopathy (NM). We found increased tension cost, and reductions in k_{tr} and calcium sensitivity in NM fibers when compared to human control fibers, consistent with the findings from nebulin-deficient murine muscle. This novel role of nebulin in regulating muscle contraction adds a new level of understanding to skeletal muscle function, and might provide a mechanism for the muscle weakness in patients with nebulin-based Nemaline Myopathy.

Platform AF: Bacterial Motility

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High Resolution, Long Term Characterization of Bacterial Motility Using Optical Tweezers

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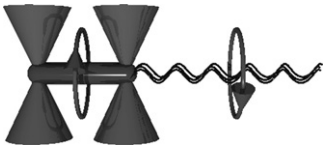

We present a single-cell motility assay[1], which allows the quantification of bacterial swimming in a well-controlled environment, for durations of up to an hour and with a temporal resolution greater than the flagellar rotation rates of ~100 Hz. The assay is based on an instrument combining optical tweezers, light and fluorescence microscopy, and a microfluidic chamber. Using this device we characterized the long-term statistics of the run-tumble time series in individual *Escherichia coli* cells. We also quantified higher-order features of bacterial swimming, such as changes in velocity and reversals of swimming direction.

[1] Min, T.L., Mears, P.J., Chubiz, L.M., Rao, C.V., Golding, I. & Chemla, Y.R. (2009) High-resolution, long-term characterization of bacterial motility using optical tweezers. *Nature Methods* (in press)

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Impact of Microscopic Motility Schemes on the Overall Swimming Behavior of Parasites

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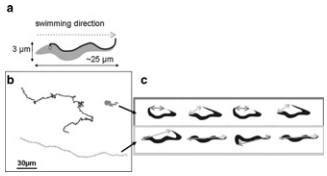

Figure 1. a) schematic of cell body b) typical swimming trajectories c) directionally persistent cells are 'stretched'

In recent work, Engstler et al. showed that the motility of the trypanosomes, causative agents of African sleeping sickness, is essential in their evasion of the host immune response. Our studies reveal that the trypanosome travels in one of three distinct motility modes: random walk, directional persistence, and an intermediate class in which they exhibit a combination of both. To further elucidate the parasite's motility we utilize high-speed videomicroscopy to uncover the microscopic origin of the macroscopic motility modes. Trypanosome swimming is facilitated by a flagellum that runs along the cell body with only a small 'free' segment at the anterior end of the cell. We use a straightforward parameter, namely the distance between the anterior and posterior ends of the cell to characterize trypanosome swimming. Remarkably this parameter is sufficient for extraction of relevant time scales for classification of the motility modes. Further, we find not only that these different motility modes correspond to distinct physical movements but also that a stiffer cell body gives rise to directional persistence.

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A Model for Bacterial Motility Utilizing Helical Cytoskeleton Filaments and Ion-Driven Motors

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The bacterial cytoskeleton determines cell shape and mediates cell division. Recent work indicates that the cytoskeleton mediates cell motility as well. Adventurous (A) motility in Myxococcus xanthus requires actin-like MreB filaments. During the motility, a double helix structure – possibly consisting of MreB – rotates in the cell's cytoplasm. Proteins localized along the helical structure are associated with proton transporting protein complexes homologous to the MotA-MotB stator that drives rotation of the bacterial flagellar motor. These observations suggest an entirely new model for bacterial motility in which motors driven by ion motive force move along the helical cytoskeleton to generate propulsive forces. This mechanism may be widespread in bacteria, since both MreB homologs and MotA-MotB homologs are common across a variety of bacterial species, including species that move in the absence of flagella. We have constructed a biophysical model to test the feasibility of this motility mechanism. Our model explains many intriguing observations in Myxococcus motility, including rotation of the helical cytoskeleton, periodic reversals of cells, and clustering of motility-related proteins at the cell poles and the substrate interface. Previous models assumed that periodic cell reversals are attributed to biochemical oscillators in the cell. This model, in contrast, proposes that reversals result from a mechanical oscillator intrinsic to a system with transmembrane motors traveling on a closed helical track. According to this mechanism, mechanical interactions play an important role in signal transduction.

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Direct Evidences of a Motility Motors in Myxococcus Xanthus

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Myxococcus xanthus, a gram-negative soil bacterium, glides over solid surfaces with two independent motility mechanisms termed social (S) and adventurous (A) motility. S-motility has been shown to be powered by the extrusion, adhesion and retraction of type IV pili. A-motility, however, is much less well understood. Two main models have been proposed to explain A-motility. The first, the ''slime gun'' model, implicates the secretion of polyelectrolyte slime from the cell pole, pushing a cell forward. More recently, we proposed a second model that involves lateral force generation at focal adhesion sites between the cell and substrate. In this model, unknown motors drive cells forward by moving along a filament inside the cell. To date, however, there has been no direct evidence for lateral force generation or the presence of motor proteins in Myxococcus.

We have developed an optical trapping bead assay, which enables us to adhere polystyrene beads to the surface of Myxococcus cells. We find that beads are moved along the cell surface with speeds comparable with those of gliding cells. Beads move in a helical pattern along the cell surface with a pitch size of a few microns. Multiple beads on the same cell can move in the different directions, indicating that beads are carried by individual motors instead of by a global movement of the cell envelope. We also show that in mutant cells lacking key A-motility regulatory genes, beads move in a less coordinated fashion.

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Morphogenesis and Cell Division of E. Coli Under Mechanical Confinement

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Bacteria have characteristic shapes and sizes which are conserved by an elaborate cytoskeletal machinery. Surprisingly, these well-defined shapes are strongly modified in E. coli bacteria in narrow nanofabricated channels. Growth in constrictions where bacteria are squeezed to about twice thinner than their typical diameter leads to flattened cells that laterally are much wider (up to 5 micron) than regular E. coli [1]. We will report on the cell growth, spatial structure and dynamics of cytoskeletal proteins and the nucleoid in this unusual bacterial phenotype. While the physical confinement has a profound effect on the cell shape and the pattern of cell division, it has only a limited effect on the replication rate of the cells. In most cases, broad (multinucleate) cells are still able to segregate chromosomes in roughly equal amounts to two daughter cells. This process typically starts with the formation of a chromosome-free area in the middle of the cell, which propagates asymmetrically to the perimeter of cell.