ventricular elastance during systole. Therefore it is paramount to identify specific sites of PTMs. In-gel digestion has classically been used for PTM identification, however this approach is limited by protein size, pI, and difficulties in peptide extraction. We report a solution-based workflow for purifying endogenous sarcomeric proteins designed to enrich for peptides containing low-abundance PTMs. We focus particular attention on regulatory light chain (RLC), which was shown first by W.T. Perrie and S.V. Perry to be phosphorylated *in vivo*, but the specific sites have been unclear. Simplification of our sample with sub-cellular fractionation followed by OFFGEL electrophoresis (OGE) resulted in discriminatory purification of thick filament proteins including regulatory and essential light chains, myosin heavy chain, and myosin binding protein-C. Digestion and HPLC profiling of OGE-separated charge variants identified unique peptides suggestive of protein modifications, thus effectively enriching for endogenous PTMs which are low in abundance and have been historically difficult to identify with mass spectrometry. In addition, UV detection provided an additional unbiased quantitative analysis of peptides without having to explore more time-intensive quantitative MS methods. Using LC/MS/MS we unequivocally identified three distinct endogenous charge variants of cardiac RLC in unique OGE fractions, thus providing explanation for isoelectric point shifts observed, both in OGE and 2D-PAGE. The singly- versus doubly-phosphorylated RLC may evoke unique conformational states and thus may be functionally distinct in regulating cardiac contraction.

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High Resolution Top-Down MS/MS Reveals Single Amino Acid Sequence Polymorphisms in Rat Cardiac Troponin

Raquel Sanchos Solis[1], Ying Ge[1], Jeffery W. Walker[2].
[1]University of Wisconsin, Madison, WI, USA, [2]University of Arizona, Tucson, AZ, USA.

Heterotrimeric cardiac troponin (cTn) is a critical component of the thin filament regulatory complex. Two of the three subunits, cTnI and cTnT, are expressed only in cardiac muscle and are widely used in the clinic as serum biomarkers of cardiac injury. cTnI and cTnT are subject to extensive post-translational modification such as proteolysis and phosphorylation, but linking modification patterns to function remains a major challenge. In order to obtain a global view of the state of post-translational modification of cTn, we are performing high resolution top-down mass spectrometry on cTn subunits isolated from native tissues. Whole cTn complexes affinity purified from a single rat heart were analyzed in a 7 Tesla Thermo LTQ-FT-ICR mass spectrometer equipped with an ESI source. High resolution MS spectra of cTn from healthy adult rats showed molecular ions for intact cTnT and cTnI as well as phosphorylation and acetylation patterns similar to human cTnI (Zabrouskov et al., 2008 *Mol Cell Proteomics*, in press). 'Shadow peaks' of similar intensity to parent peaks were detected exhibiting masses of cTnI + 16 Da and cTnT + 128 Da, suggestive of single amino acid polymorphisms. Tandem mass spectrometry (MS/MS) analysis by ECD and CAD fragmentation of intact and protease-digested cTn subunits localized an Ala/Ser polymorphism at residue 7 of cTnI, and an additional Gln within a 3 residue alternative splice site beginning at residue 192 of cTnT. High resolution top-down MS/MS has revealed intriguing heterogeneity not only in the extent of phosphorylation, but also in amino acid sequences of cTnI and cTnT even within a single rat heart. Supported by NIH, AHA, UW-CVRC & Wisconsin Partnership for a Healthy Future.

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Cytotoxicity of non-myofilament-incorporated troponin T fragments

Euy-Myoung Jeong, M. Moazzem Hossain, J.-P. Jin.
NorthShore University Health System and Northwestern University Feinberg School of Medicine, Evanston, IL, USA.

Troponin T (TnT) is a striated muscle-specific protein and an abundant component in skeletal and cardiac myofilaments. Forced expression of TnT protein in non-muscle cells or undifferentiated myoblasts in the absence of myofibrils indicated cytotoxicity (Wang *et al., J. Biol. Chem.* 280:13241-9, 2005). To investigate the cytotoxic effect of non-myofibril-incorporated TnT, we constructed non-fusion co-expression vectors encoding green fluorescence protein tracer and different regions of the TnT polypeptide chain. Transient transfection in culture was studied in HEK293 non-muscle cells and undifferentiated C_2C_{12} myoblasts. Cytotoxicity of the TnT fragments was examined by the viability of the transfected cells. The results revealed distinct toxic effects of different regions of TnT. The evolutionarily conserved middle and C-terminal segments of TnT were highly toxic to cells whereas the N-terminal variable region was not. The cytotoxicity of the middle and C-terminal regions of TnT was associated with apoptotic cell death. Although muscle cells have high capacity of proteolysis to rapidly remove non-myofilament incorporated TnT protein, peak releases of TnT or TnT fragments from myofibrils may occur in the events of myocardial ischemia reperfusion and skeletal muscle fatigue or injuries. When the level of non-myofilament-associated TnT and TnT fragments exceeds the protective capacity of proteolytic removal in the muscle cell, they may impose cytotoxic effect and cause cell death. Therefore, the activity of non-myofilament-associated TnT or TnT fragments in inducing apoptosis and cell death is a potential pathogenic factor, particularly important in adult cardiac myocytes that lack the ability of regeneration.

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Proximity mapping of troponin T and troponin I in cardiac troponin using molecular dynamics simulations

Jayant J. Jayasundar[1], Jun Xing[2], John M. Robinson[2], Herbert C. Cheung[2], Wen-Ji Dong[1].
[1]The School of Chemical Engineering and Bioengineering and The Department of Veterinary and Comparative Anatomy Pharmacology and Physiology, Washington state university, Pullman, WA, USA, [2]The Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL, USA.

The intersite distances from cTnT residues 240, 276, and 288 to cTnI N-terminal residues 5, 17, 27, 40, and to the cTnI C-terminal residues 131, 145, 152, 160, and 167 in reconstituted cTn were determined in the presence of Mg^{2+} and Mg^{2+} plus Ca^{2+} using FRET. The distances from cTnT residues 276 and 288 to cTnI residues 5, 17, 27 were also determined in the presence of Ca^{2+} with cTnI bisphosphorylated at Ser^{23} and Ser^{24}. The results showed that the transition of troponin from the Mg^{2+} state to the Ca^{2+} state was accompanied by small to moderate changes in distances, suggesting small global conformational changes. The distance changes were accompanied by changes in the half-width of the distributions of the distances. To clarify the structural basis for population broadening, we performed MD simulations with explicit solvent. The published NMR structure of the N-terminal region of cTnI was docked and integrated into cTn. The average ensemble structure showed interactions of the cTnI N-terminal region with cTnC. In the Mg^{2+} state, the cTnI N-terminal segment interacted with the defunct Ca^{2+}-binding site I and the functional site II in cTnC. In the Ca^{2+}state, non-phosphorylated cTnI interacted with the helix A and site I of cTnC. These interactions stabilized the open hydrophobic pocket in the N-domain of cTnC, and the cTnI regulatory region was constrained within the hydrophobic pocket. The bisphosphorylated segment of cTnI was bent, interrupting its interaction with Ca^{2+} site I of cTnC. This loss of interaction resulted in depressed opening of the cTnC N-domain, forcing the regulatory region of cTnI to move out from the hydrophobic pocket.

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The Rate Of Calcium Dissociation From The Cardiac Thin Filament Is Affected By Multiple Modulatory Factors

Bin Liu[1], Sean C. Little[1], Ryan S. Lee[1], Kristopher P. Kline[1], Darl R. Swartz[2], Svetlana B. Tikunova[3], Jonathan P. Davis[1].
[1]The Ohio State University, Columbus, OH, USA, [2]Purdue University, West Lafayette, IN, USA, [3]University of Houston, Houston, TX, USA.

The rate of cardiac muscle relaxation is dynamically modulated. We hypothesize that the rate of Ca2+ dissociation from the thin filament is at least one of the factors that can strongly influence the rate of relaxation. Thus, we would expect the various physiological and pathological stimuli that affect the kinetics of cardiac muscle relaxation to likewise affect the rate of Ca2+ dissociation from the thin filament. In this study, we investigated various modulators of the Ca2+ exchange kinetics in the physiologically relevant biochemical model systems of reconstituted thin filaments and rabbit ventricular myofibrils such as: 1) PKA phosphorylation of TnI, 2) ischemia-reperfusion associated truncation of TnI, 3) familial cardiomyopathy related mutations of TnI and TnT, 4) the calcium sensitizing compound bepridil, 5) rationally engineered TnC mutations, and 6) tropomyosin isoforms. Consistent with the effects of PKA on accelerating relaxation, the rate of Ca2+ dissociation from the thin filament was accelerated by TnI mutations (S23D,S24D) mimicking PKA phosphorylation. Additionally, the rate of Ca2+ dissociation was slowed by truncation of TnI (residues 1-192), consistent with ischemia-reperfusion slowing the rate of cardiac relaxation. The hypertrophic and restrictive cardiomyopathy mutations (TnIS166F and TnIR192H) slowed the rate of Ca2+ dissociation from