its >6000 anti-human polyclonal, affinity-purified antibodies of which 49 were identified by MS and 79 were novel. HPA antibodies were available for 82 the 142 proteins but only 43 reacted positively with the ICDs based on immunohistochemistry. We supplement these approaches with 14 proteins identified using conventional 2-DE. Combining all techniques. Only 14 proteins were common to the MS data, HPA antibodies and the literature. We can categorize all 273 identified ICD proteins according to their known functions and demonstrate their functional interactions in a single inter-active relationship according to their functions: (i) adhesion, anchoring and binding (88); (ii) enzymes (46); (iii) proteins that maintain the structure and function of the ICDs (35); (iv) myofibrillar (34); (v) channels (32); (vi) ligands and their receptors (18); (vii) cytoplasm proteins (6); and (viii) mechanoreceptors (4). We will now extend these analyses to ICD proteins that change as a result of human heart failure.

3933-Plat

Quantitative Analysis of MyBP-C Phosphorylation in Human Heart using Phosphate Affinity SDS-Page

Steven Marston[1], Sakthivel Sadayappan[2], O'neal Copeland[1].
[1]Imperial College London, London, United Kingdom, [2]Loyola University Chicago, Chicago, IL, USA.

Phosphorylation sites in Cardiac MyBP-C have been predicted at Ser273,282 and 302 but studies in intact tissue have identified 5 phosphorylted sites and suggested up to 4.6molsPi/mol MyBP-C is present in human heart.

We analysed MyBP-C phosphospecies in human heart myofibrils by phosphate affinity SDS-PAGE using a non-specific antibody raised against the MyBP-C peptide 2-14. We observed six bands corresponding to 0, 1P, 2P, 3P, 4P, 5P phospho-species. Control experiments with pure MyBP-C indicated that the antibody labelled all phosphospecies equally. The assigned phosphorylation levels were confirmed by staining western blots with PhosTools phosphoprotein stain. This separation permits direct quantitative determination of MyBP-C phosphospecies without need for calibration.

In donor heart myofibrils the highly phosphorylated species predominated: 0, $7 \pm 3\%$: 1P, $1 \pm 1\%$: 2P, $23 \pm 7\%$: 3P, $41 \pm 2\%$: 4P, $20 \pm 8\%$ (n=4) from which total phosphorylation of MyBP-C was calculated to be 3.4molsPi/mol. In failing heart unphosphorylated MyBP-C predominated (0, $48 \pm 4\%$: 1P, $4 \pm 4\%$: 2P, $27 \pm 5\%$: $1 \pm 1\%$: 3P, $17 \pm 4\%$: 4P, $4 \pm 2\%$, n=4) and calculated total phosphorylation was 1.5 molsPi/mol. Total phosphorylation in failing heart myofibrils was 44% of donor and in myectomy samples from HCM patients it was 29% of donor, compared with 45 and 40% respectively determined in previous assays.

We conclude that MyBP-C is highly phosphorylated in vivo with significant phosphorylation of at least 5 sites and that phosphorylation is dynamic, being greatly reduced in pathological muscle. Initial tests using antibodies specific to Ser 273, 282 and 302 show distinct patterns on phosphate affinity SDS-PAGE indicating varying preferences for the highly phosphorylated species of MyBP-C in normal and pathological muscle.

3934-Plat

Identification of Amino Acid Residues in the Cardiac Myosin Binding Protein-C Motif Important for Actin Binding

Justin F. Shaffer[1,2], Samantha P. Harris[2].
[1]University of Washington, Seattle, WA, USA, [2]University of California, Davis, Davis, CA, USA.

N-terminal domains of cardiac myosin binding protein-C (cMyBP-C) can activate actomyosin interactions in the absence of Ca^{2+} and bind to actin in a phosphorylation dependent manner. We have previously shown that two N-terminal domains, C1 and the MyBP-C motif (''M'') domain, bind specifically to actin and to thin filaments; however, the sequences or residues that mediate actin binding have not been identified. The goal of this study was to identify residues in the M-domain that contribute to actin binding and to investigate whether interactions between the M-domain and actin mediate the activating properties of cMyBP-C. We therefore used alanine-scanning mutagenesis to target candidate actin binding sites in the M-domain that bear homology to the actin binding motifs in other known actin binding proteins and to assess the effects of mutations on the ability of recombinant proteins to bind actin and activate actomyosin interactions in motility assays. Results demonstrate that mutation of select positively-charged amino acids in the M-domain that are homologous to binding motifs in known actin binding proteins reduced binding of cMyBP-C to actin. The mutations also reduced or eliminated the activating properties of recombinant cMyBP-C in *in vitro* motility assays. However, mutation of other positively-charged amino acids did not affect actin binding or protein functional properties. These results indicate that specific residues within the M-domain confer actin binding and that interactions with actin contribute to the functional effects of recombinant cMyBP-C N-terminal proteins. Supported by NIH HL080367 to SPH and a NSF Graduate Research Fellowship to JFS.

3935-Plat

N-Terminal Fragments of Cardiac Myosin Binding Protein-C Inhibit Actomyosin Motility by Tethering Actin

Abbey E. Weith[1], Sakthivel Sadayappan[2], Peter VanBuren[1], Jeffrey Robbins[2], David M. Warshaw[1].
[1]University of Vermont, South Burlington, VT, USA, [2]Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA.

Cardiac myosin binding protein-C (cMyBP-C) mutations are a leading cause of hypertrophic cardiomyopathy. cMyBP-C has 11 domains, C0 through C10, that bind sarcomeric proteins, including myosin and actin. A 29 kD N-terminal fragment (C0C1f) of cMyBP-C containing the first two domains C0 and C1 and the first 15 residues of the conserved MyBP-C motif is cleaved from cMyBP-C following ischemic-reperfusion injury (Sadayappan et al., JMCC 44:S44, 2008). Expressed C0C1f fragments inhibit actin velocities in the motility assay at a 2:1 molar ratio to myosin, similar to other N-terminal fragments: C0C3, C0C2, and C1C2. Interestingly, fragments containing only the C0C1 domains do not alter velocity, suggesting the additional 15 residues in C0C1f are necessary for inhibition. Adding C0C1 to the motility assay can partially reverse the C0C3-mediated inhibition of velocity, suggesting C0C1 may compete with C0C3 for actin binding. cMyBP-C fragments may affect motility by creating a tether between actin and the flowcell surface. To test this, motility experiments were performed under high ionic strength, saturating MgATP, and in the absence of methylcellulose, conditions in which most actin filaments diffuse away from the surface due to weak interactions with myosin. In the presence of C0C2, many actin filaments bound and translocated on the surface, confirming this fragment's tethering capacity. Additionally, in the laser trap we adhered C0C3 fragments to a bead in the absence of myosin and observed C0C3 transiently binding to a single actin filament with an ~100 ms attached lifetime. We also saw evidence that C0C3 may partially unfold under load. These experiments strongly suggest that N-terminal domains of cMyBP-C containing the MyBP-C motif tether actin filaments and provide one mechanism for modulating actomyosin motion generation, i.e. by imposing an effective viscous load within the sarcomere.

3936-Plat

PKC Phosphorylation of Titin's PEVK Element–A Novel and Conserved Pathway for Modulating Myocardial Stiffness

Bryan D. Hudson[1], Carlos G. Hidalgo[1], Julius Bogomolovas[2], Yi Zhu[1], Brian Anderson[1], Marion Greaser[3], Siegfried Labeit[2], Henk L. Granzier[1].
[1]University of Arizona, Tucson, AZ, USA, [2]Universitatsmedizin, Mannheim, Germany, [3]University of Wisconsin, Madison, WI, USA.

Protein Kinase C (PKC) regulates contractility of cardiac muscle cells by phosphorylating multiple thin- and thick- filament based proteins, and plays key roles in development of cardiovascular pathologies. Myocardial sarcomeres also contain a third myofilament, titin, which we demonstrate here to also be phosphorylated by PKC. Titin phosphorylation was observed in skinned myocardial tissues following incubation with PKCα and this effect was exacerbated ~5 fold in the mouse and ~2.5 fold in the pig by preincubation with Protein Phosphatase 1 (PP1). In vitro phosphorylation of recombinant protein representing titin's spring elements shows that PKCα targets the PEVK spring element. Mass spectrometry in combination with site-directed mutagenesis identified two highly conserved sites in the PEVK region that are phosphorylated by PKCα (S11878 and S12022); when these two sites are mutated to alanine, phosphorylation is effectively abolished. Mechanical experiments with murine and porcine skinned LV myocardium revealed that PKCα significantly increases titin-based passive tension in a sarcomere length (SL)-dependent fashion. Single molecule force-extension curves show that PKCα decreases the PEVK persistence length (from 1.20 nm to 0.55 nm), without altering the contour length, and using a serially-linked wormlike chain (WLC) model we show that this results in an ~20% increase in titin-based passive force with a SL dependence that is similar to that measured in skinned myocardium following PKCα phosphorylation. We conclude that PKC phosphorylation of titin is a novel and conserved pathway that links myocardial signaling and myocardial stiffness.

3937-Plat

Titin Strucure and Extensibility in Healthy and Failing Hearts

Helen K. Graham, Michael J. Sherratt, Andrew W. Trafford.
University of Manchester, Manchester, United Kingdom.

The giant sarcomeric protein, titin is the primary determinant of myocardial passive stiffness. In failing human hearts however, it has been proposed that reduced myofilament passive tension is due to an altered titin isoform expression profile[1, 2]. In this study we set out to directly quantify the tensile strength of titin molecules isolated from healthy and diseased myocardium.