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ONLINE SUPPLEMENT

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This supplemental material provides the Material and Methods used for mass spectrometry identification of cMyBP-C phosphorylation status in the NTG mouse. Figures regarding results of mass spectrometry are presented here (Supplemental Figure 1-2). Also, the figures that describe the X-ray diffraction intensity profiles for all four populations under all three conditions examined (Supplemental Figure 3-4).

Materials and Methods

Quantification of site-specific endogenous cMyBP-C phosphorylation in NTG mice: Four specific amino acids residues within cMyBP-C isolated from NTG mice were determined to contain phosphate by electron ionization (ESI) liquid chromatography-tandem mass spectrometry (LC-MS/MS). The degree of phosphorylation at each site was quantified by label-free LC-MS/MS using a mass balance approach (6). This approach determines the degree of phosphorylation in a sample by comparing the abundance of peptides that were altered by phosphorylation to a control sample devoid of phosphorylation.

In preparation for LC-MS, myofibrillar proteins were extracted from the apex of mouse hearts and digested to peptides using trypsin. Small ~10 mg pieces of cardiac muscle were visualized through a dissecting microscope and teased into ~1 mm strips using forceps while soaking in chilled relaxing solution containing, 50 mM NaCl, 2 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, 7mM phosphate buffer (pH 7), 2.5 mM ATP, and 10 mM creatine phosphate. The strips of muscle were transfered into chilled relaxing solution containing 0.5% Triton X-100 (Sigma) and dissected further to ~0.5 mm. The muscle strips were transferred again into fresh, chilled relaxing solution containing 0.5% Triton X-100 to remove the membrane proteins and subsequently transferred two more times to fresh, chilled relaxing solution to remove residual Triton X-100. Protein digestion was carried out by adding 10-15 of the resultant ~0.5 mm muscle strips to a 1 mL Eppendorf tube containing 2 µg of sequencing grade modified trypsin (Promega) in 100 µL of 50 mM ammonium bicarbonate. The tubes were incubated at 30 °C for 18 h in a heating block, and periodically agitated during the incubation to aid in the dispersion and digestion of the proteins in the muscle strips. The trypsin was inactivated with the addition of 7.5 µl of 90% formic acid, and the tubes were dried in a speed vacuum device. The resultant peptides were reconstituted in 30 µl of 0.05% heptafluorobutyric acid (HFBA). The HFBA was used to enhance the retention of hydrophilic peptides (1) that can be lost in the void volume of the LC injection.

Liquid chromatography was performed using a 100 mm x 1mm I.D. BioBasic column packed with 5 µm C18 (Thermo Electron Corporation) and protected by a 4 mm x 1mm I.D. SecurityGuard C18 column (Phenomenex). The columns were connected to a Shimadzu MS pump and autosampler (Shimadzu Biotech). Each sample was injected into 0.5% formic acid in 5.0% acetonitrile at a 25 µl/min flow rate. At 4 minutes, the flow was increased to 50 µl/min and the gradient was ramped linearly to 0.5% formic acid in 45% acetonitrile over 30 min and held isocratic for 10 min. The ramp was then increased to 0.5% formic acid in 60% acetonitrile for 6 min and then returned to 0.5% formic acid in 5% acetonitrile. The column was allowed to re-equilibrate for 30 min prior to the next injection. The total run time was 86 min per analysis.

The LC eluant entered a LTQ ion trap mass spectrometer (Thermo Electron Corporation) by direct coupling with an ESI interface. The instrument was operated in positive ESI mode with a capillary temperature of 275 °C and a spray voltage of 5.0 kV. Mass

spectra were collected in data dependent mass spectrometry-mass spectrometry (MS/MS) mode. The instrument cycled between scanning for peptides with an m/z ratio of 260-2000 Da using automatic gain control and collecting data dependant MS/MS spectra of fragmented peptides. One data dependant MS/MS analysis was performed per MS scan when an ion in the MS scan produced a minimum MS signal of $5x10^3$. An isolation width of 1, collision energy of 35%, activation Q of 0.25, activation time of 30 ms, and a repeat count of 2 with 60 second exclusion were used for each MS/MS analysis. The MS and MS/MS spectra were collected in individual ".raw" data files for each sample.

Peptides were identified from the MS/MS spectra contained in the ".raw" files using the automated search algorithm SEQUEST (3) and the identifications were manually confirmed. SEQUEST searches were performed via BioWorks software (Thermo-Finnigan) using a search library constructed to contain only the mouse cMyBP-C (O70468) sequence downloaded from UniProtKB (4) through www.expasy.org (5). Methionine oxidation was accounted for by allowing for the differential addition of 16.0 and 32.0 Da to each methionine residue, for one and two oxygen atoms. Peptide phosphorylation was accounted for by allowing for the differential addition of 80.0 Da to each serine, threonine, and tyrosine residue. The MS/MS spectra of peptides identified using SEQUEST were compared to theoretical spectra generated using Protein Prospector MS-Product (http://prospector.ucsf.edu/ucsfhtml4.0/msprod.htm), ensuring a continuum of y- and b-ions unique to each peptide sequence was present in each spectrum.

LC elution profiles were generated for each peptide of interest by extracting the $[M+H]^+$ or $[M+2H]^{2+}$ ion currents from the MS spectra in the ".raw" data files. Ion current peaks corresponding to each peptide contained a minimum of 30 data points. The area under each peak was integrated using Xcalibur (ThermoFisher Scientific), recorded in Excel (Microsoft), and used to determine the percent phosphorylation.

Results and Data analysis

The six phosphorylated peptides listed in Supplemental Table 1 were identified in MS/MS spectra from the NTG mice. Supplemental Figure 1 shows an example of the MS/MS spectra for the $^{271}RTS_pLAGAGR^{279}$ phosphorylated peptide. As illustrated in Supplemental Figure 1, the most intense peak in the MS/MS spectra from the phosphorylated peptides corresponds to the mass of the phosphorylated peptide minus the neutral loss of phosphate (98.0 Da for $[M+H]^+$ and 49.0 Da for $[M+2H]^{2+}$). A continuum of y- and b-ions unique to each peptide sequence is present in each spectrum at a lesser abundance than the neutral loss ions, but the intensity of the y- and b-ions is still well above the background noise.

Several of the phosphorylated peptides listed in Supplemental Table 1 contain homologous amino acid sequences but have "ragged ends" resulting from differential tryptic cleavage due to the presence of phosphate near the cleavage site. The six phosphorylated peptides collectively contain four unique phosphorylated amino acid residues; serine 273, 282, 302, and 307. Although these phosphopeptides were observed in the endogenous cMyBP-C, the percent phosphorylation at each site cannot be directly determined from the abundance of the ion currents of the phosphopeptides in the MS spectra because the digestion and ionization efficiencies of each phosphopeptide are unknown. We therefore developed a mass balance approach that indirectly calculates the percent phosphorylation at each site within the endogenous cMyBP-C from the "expected abundance" of non-phosphorylated peptides that include the phosphorylatable amino acids when the percent phosphorylation equals zero.

Supplemental Table 1. Phosphorylated peptides observed and degree of phosphorylation determined for cMyBP-C isolated for NTG mice.

Phosphorylated Amino Acid	Phosphopeptide Observed	m/z	Phosphorylation (% ± SD)
Serine 273	272TSpLAGAGR279	406.7	**76 ± 11**
	271RTSpLAGAGR279	484.7	
Serine 282	281TSpDSHEDAGTLDFSSLLK298	1002.0	**100 ± 0**
	280RTSpDSHEDAGTLDFSSLLK298	1080.0	
Serine 302	299KRDSpFR304	444.7	**31 ± 12**
Serine 307	305RDSpKLEAPAEEDVWEILR322	1118.5	**11 ± 3**

The "expected abundance" of the non-phosphorylated peptides including the phosphorylatable amino acids was directly determined from the analyses of a second set of samples that did not contain any phosphorylation. Rather than relying on the dephosphorylation of the endogenous cMyBP-C, we utilized a protein fragment corresponding to amino acids 1-539 of mouse cMyBP-C (domains C0-C3) that was bacterially expressed from mouse cardiac cDNA using a pET expression system (Novagen, Madison, WI) (2). Two 2 μg aliquots of the expressed protein were digested and prepared for LC-MS analysis as described above for the muscle strip containing the endogenous mouse cMyBP-C.

The ion currents for the properly cleaved non-phosphorylated peptides containing serines 273, 282, and 302, (I_X) being ^{272}TSLAGAGR^{279} (m/z = 366.9), ^{281}TSDSHEDAGTLDFSSLLK^{298} (m/z = 962.2), and ^{301}DSFR^{304} (m/z = 524.3) were observed in the expressed C0c3 control samples and the "measured abundance" of each peptide was recorded. Although the ^{306}DSK^{308} (m/z = 349.2) peptide containing serine 307 was identified in the MS/MS spectra, the peptide eluted in the void volume of the column (retention time < 2 min) with several other peaks of similar mass. We therefore recorded the "measured abundance" of the neighboring ^{309}LEAPAEEDVWEILR^{322} (m/z = 835.8) peptide that was included in both differentially cleaved phosphopeptides containing serine 307. Because the "measured abundance" of each peak from the MS spectra scales with the total amount of protein digested, the "expected abundance" of the ^{272}TSLAGAGR^{279}, ^{281}TSDSHEDAGTLDFSSLLK^{298}, ^{301}DSFR^{304} and ^{309}LEAPAEEDVWEILR^{322} peptides was determined as a ratio of the measured abundance of these peptides to the measured abundance of reference peptides that are not affected by phosphorylation from within the C0C3 samples. The use of five independent reference peptides (I_R) ^{55}YGLAAEGK^{62} (m/z = 405.0), ^{94}VTEPAPPEK^{102} (m/z = 484.6), ^{176}VAGASLLKPPVVK^{188} (m/z = 640.0), ^{406}YIFESVGAK^{414} (m/z = 507.7), and ^{485}DGVELTR^{491} (m/z = 395.5) for determining the I_X/I_R ratios minimizes variability in the digestion efficiency and LC-MS measurement.

Once the expected abundance of the of the non-phosphorylated peptides was determined from the control samples $(I_X/I_R)_{control}$ the percent phosphorylation at serine 273, 282, 302, and 307 in the endogenous cMyBP-C samples was indirectly determined using:

Equation 1

$$X_p = \left[1 - \frac{(I_X / I_R)_{\text{Endogenous MyBP-C}}}{(I_X / I_R)_{\text{control}}} \right] * 100$$

To increase the accuracy and precision of our measurement, the percent phosphorylation of each serine was independently determined from each of the five reference peptides in the

sample and then averaged. We report the arithmetic mean and standard deviation of the percent phosphorylation calculated from the duplicate endogenous cMyBP-C samples and LC-MS analyses.

The percent phosphorylation determined for serines 273, 282, 302, and 307 in the PKA treated samples is listed in Supplemental Table 1. As a graphical example of the quantification, the relationship between the ion current for ^{281}TSDSHEDAGTLDFSSLLK^{298} peptide, differentially cleaved ^{281}TS$_{p}$DSHEDAGTLDFSSLLK^{298} and ^{280}RTS$_{p}$DSHEDAGTLDFSSLLK^{298} phosphopeptides, and ^{406}YIFESVGAK^{414} reference peptide in the control and endogenous cMyBP-C samples is shown in Supplemental Figure 2. Endogenous phosphorylation of cMyBP-C resulted in a 100% reduction in the abundance of the ^{281}TSDSHEDAGTLDFSSLLK^{298} peptide indicating that serine 282 is 100% phosphorylated. Moreover, the relative abundance of the properly cleaved ^{281}TS$_{p}$DSHEDAGTLDFSSLLK^{298} phosphopeptide was only ~60% that of the miscleaved ^{280}RTS$_{p}$DSHEDAGTLDFSSLLK^{298} phosphopeptide including the additional R^{280}, illustrating the long ranged inhibitory effect of phosphate on tryptic cleavage when phosphate is located near the cleavage site.

Supplemental Figure Legends

Supplemental Figure 1. A MS/MS spectrum from the phosphorylated $^{271}RTS_{p}LAGAGR^{279}$ peptide observed in the samples from the NTG mice. Fragmentation of the $[M+2H]^{2+}$ parent ion, m/z = 484.7, produced daughter ions including those with the neutral loss of 98 corresponding to m/z = 49 for $[H_3PO_4]^{2+}$, 17 corresponding to m/z = 8.5 for $[NH_3]^{2+}$, and 116 Da corresponding to m/z = 58 for $[H_3PO_4 + H_2O]^{2+}$. The most intense peak in the spectrum (m/z = 435.9) corresponds to the neutral loss of phosphate and is off-scale at 100%. The y-axis was scaled to 12% to show the y- and b-ions used to confirm the identification of the peptide sequence.

Supplemental Figure 2. LC-MS peptide elution profiles generated by extracting the $[M+2H]^{2+}$ ion currents for the $^{281}TSDSHEDAGTLDFSSLLK^{298}$ (black, m/z = 962.2) $^{281}TS_{p}DSHEDAGTLDFSSLLK^{298}$ (blue, m/z = 1002.0) ,$^{280}RTS_{p}DSHEDAGTLDFSSLLK^{298}$ (red, m/z = 1080.0), and $^{406}YIFESVGAK^{414}$ (grey, m/z = 507.7) peptides from a (A) expressed C0C3 control and (B) endogenous cMyBP-C sample. In A and B, the intensity of each peak is normalized to the intensity of the peak for the $^{406}YIFESVGAK^{414}$ reference peptide and set to 100,000. A) Only peaks corresponding to the non-phosphorylated $^{281}TSDSHEDAGTLDFSSLLK^{298}$ and $^{406}YIFESVGAK^{414}$ reference peptides are observed in the contorl sample. B) The samples from the NTG mice only contain the differentially cleaved $^{281}TS_{p}DSHEDAGTLDFSSLLK^{298}$ and $^{3280}RTS_{p}DSHEDAGTLDFSSLLK^{298}$ phosphopeptide. The $^{281}TSDSHEDAGTLDFSSLLK^{298}$ peptide is not present in the cMyBP-C samples from the NTG mice due to the inclusion of this peptide in the two phosphopeptides.

Supplemental Figures 3 and 4. Small-angle X-ray diffraction detection of myofilament lattice spacing. **A and B**. Under relaxed conditions (green), the diffraction peaks indicating $d_{1,0}$ and $d_{1,1}$ profiles of t/t and AllP-$_{t/t}$ (Panel B) were broader and weaker compared to NTG and $WT_{t/t}$ (Panel A). These characteristics are consistent with increased paracystalline disorder in the myofilament lattice. Under activated conditions (red), the $d_{1,0}$ and $d_{1,1}$ peaks still demonstrated greater disorder in the t/t and AllP-$_{t/t}$ compared to NTG and $WT_{t/t}$. Under rigor conditions (black), the intensity of the $d_{1,1}$ tended to increased compared to that of $d_{1,0}$. In the t/t and AllP-$_{t/t}$, the peaks under rigor conditions also tended to move to higher angles of diffraction compared to relaxed and activated conditions, which indicated a relative compression of the myofilament lattice in these populations.

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