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Identification of Novel Protein Kinase A Phosphorylation Sites in the M-domain of Human and Murine Cardiac Myosin Binding Protein-C Using Mass Spectrometry Analysis

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Abstract

Cardiac myosin binding protein-C (cMyBP-C) is a large multi-domain accessory protein bound to myosin thick filaments in striated muscle sarcomeres. It plays an important role in the regulation of muscle contraction and mutations in the gene encoding cMyBP-C are a common cause of familial hypertrophic cardiomyopathy, the leading cause of sudden cardiac death in young people[1]. The N-terminal domains including the C0, C1, cMyBP-C motif, and C2 domains play a crucial role in maintaining and modulating actomyosin interactions (keeping normal cardiac function) in a phosphorylation dependent manner. The cMyBP-C motif or “M-domain” is a highly conserved linker domain in the N-terminus of cMyBP-C that contains three to five protein kinase A (PKA) phosphorylation sites, depending on species. For the human isoform, three PKA sites were previously identified (Ser^{275}, Ser^{284}, and Ser^{304}), while three homologous sites exist in the murine isoform (Ser^{273}, Ser^{282}, and Ser^{302}). The murine cMyBP-C isoform contains an additional conserved consensus site, Ser^{307} that is not present in the human isoform. In this study, we investigated sites of PKA phosphorylation of murine and human cMyBP-C by treating the recombinant protein C0C2 (~50 KDa, which contains the N-terminal C0, C1, M, and C2 domains) and C1C2 (~35 KDa, contains C1, M and C2 domains) with PKA and assessing the phosphorylation states using SDS-PAGE with ProQ Diamond staining, and powerful hybrid mass spectrometric analyses. Both high-accuracy bottom-up and measurements of intact proteins mass spectrometric approaches were used to determine the phosphorylation states of C0C2 and C1C2 proteins with or without PKA treatment. Herein, we report for the first time that there are four PKA phosphorylation sites in both murine and human M-domains; both murine Ser^{307} and a novel

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Supporting Information Available: Supplementary materials containing more detailed technical information of methods, sequence information of proteins, schematic diagram of various species of cMyBP-C M-domains, more MS^2 and MS^3 spectra of identified phosphorylation sites, and more mass spectra of intact proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

human Ser^{311} can be phosphorylated *in vitro* by PKA. Future studies are needed to investigate the phosphorylation state of murine and human cMyBP-C *in vivo*.

Keywords

cardiac myosin binding protein-C; phosphorylation; mass spectrometry; bottom-up; measurement of intact protein; LTQ-Orbitrap

Introduction

Myosin binding protein-C (MyBP-C)[1] is an accessory protein of vertebrate striated muscle that regulates actomyosin interactions and influences the rate and power of muscle contraction.[2, 3] Mutations in the gene encoding the cardiac (c) isoform of MyBP-C are a major cause of hypertrophic cardiomyopathy, a leading cause of sudden cardiac death in the young and of heart failure in older populations.[1, 4, 5] cMyBP-C also contributes to cardiac contractility on a beat-to-beat basis and is phosphorylated in response to inotropic stimuli, including β-adrenergic agonists that activate protein kinase A (PKA).[6] PKA phosphorylates cMyBP-C on at least three serine residues (Ser^{275}, Ser^{284}, and Ser^{304} (human numbering)) clustered within a unique regulatory sequence referred to as the MyBP-C "motif" or "M-domain" (Figure S1). Phosphorylation of cMyBP-C by PKA speeds actomyosin interactions and contributes to increased cardiac contractility following β-adrenergic stimulation.[7, 8] Phosphorylation by PKA is essential for proper cardiac function because cMyBP-C transgenic mice with selected phosphorylation sites blocked (serine to alanine substitutions)[7, 9, 10] show cardiac dysfunction and develop hypertrophy. In humans, reduced cMyBP-C phosphorylation is also associated with cardiac hypertrophy and heart failure.[11, 12] Conversely, phosphorylation of cMyBP-C appears cardioprotective because transgenic mice that constitutively express phosphorylated cMyBP-C (serine to aspartic acid substitutions) show normal cardiac function and survive ischemic challenges better than wild type mice.[13]

Although the significance of cMyBP-C phosphorylation to cardiac health is becoming increasingly apparent, the precise sites and stoichiometry of phosphate incorporation within cMyBP-C have not yet been completely characterized and species-specific differences are likely to exist. For instance, PKA treatment of bovine cMyBP-C resulted in the incorporation of 5 mol Pi/mol of cMyBP-C,[14] whereas 3 mol Pi were incorporated into chicken cMyBP-C.[15] For human cMyBP-C, PKA treatment of overlapping recombinant fragments that spanned the entire protein was consistent with 3 mol Pi/mol as determined by nano-electrospray mass spectrometry.[16, 17] However, recent studies of human donor hearts showed a total level of 4.6 ± 0.6 mol Pi/mol cMyBP-C phosphorylation,[11] suggesting that additional sites beyond the three serine residues are phosphorylated by kinases *in vivo*.

Additional phosphorylation sites within the M-domain were recently described for rat and canine cMyBP-C using MS/MS methods. For rat, one additional serine was found phosphorylated downstream of the three canonical sites identified in human[18], whereas three additional serine residues were phosphorylated in dog heart following myocardial stunning[19]. Although the specific kinase(s) responsible for phosphorylation of these serine residues were not identified, their consensus sequences are completely conserved in mouse cMyBP-C and to a lesser extent in the human isoform (Figure S1). The latter observations raise the possibility that these sites are utilized in mouse or human cMyBP-C. Although a recent mass spectrometric analysis of baculovirus expressed murine cMyBP-C did not find evidence for phosphorylation at these sites, the cMyBP-C samples were not treated with PKA prior to analysis.[20]

Mass spectrometry is a powerful tool for the identification of proteins and the characterization of variable protein posttranslational modifications (PTMs).[21-26] Electrospray ionization mass spectrometry has played an important role in the analysis of PTMs, and with additional HPLC, is capable of measuring the mass of macromolecules with high accuracy.[22, 27, 28] Currently, there are two general complementary approaches for the mass spectrometry analysis of proteins;[29-31] the bottom-up approach where proteolytic peptides and PTM sites are identified via the tandem MS and/or multistage activation strategy,[22, 32, 33] and the top-down approach which involves the analysis of intact proteins.[28, 34-36] Sometimes, the bottom-up method is limited because the peptides containing the PTMs are lost during the separation step.[29, 30, 37, 38] As a complementary method, the proteins can be identified using the top-down approach or solo mass measurements of intact proteins[28, 37] thus providing global PTMs information.

The purpose of the present study was to characterize the phosphorylation state of both the murine and human cMyBP-C M-domains in response to PKA using a hybrid approach, which utilizes both bottom-up approach and measurements of intact proteins thus increasing our confidence of protein identification and PTM characterization. Nanospray (nano-) LC-MS/MS analysis of tryptic digests of recombinant proteins containing cMyBP-C N-terminal domains using an LTQ-OrbitrapXL mass spectrometer was used to identify phosphorylation sites within the M-domain of cMyBP-C after PKA treatment. Due to the low abundance (sub-stoichiometric amounts) of protein phosphorylation and the low ionization efficiency of phosphopeptides in the commonly used positive-ion mode of mass spectrometry,[32, 39] enrichment of phosphopeptides using IMAC[40] and/or titanium dioxide[41] was used prior to their characterization. In the second approach, a high mass bench top orthogonal acceleration TOF type instrument was used to analyze the accurate mass of intact cMyBP-C N-terminal recombinant proteins to validate the observed phosphorylation sites. A biochemical mutation study (Ser to Asp) in murine cMyBP-C clearly demonstrated the discovery of a fourth PKA phosphorylation site. Our results indicate that PKA phosphorylates up to four sites in both the murine and human M-domains including a novel site not previously described for either protein (Ser^{307} for mouse and Ser^{311} for human). These additional sites may contribute to the normal physiological function of cMyBP-C.

Materials and Methods

Chemicals and materials

Ammonium bicarbonate, ammonium acetate, DL-dithiothreitol (DTT), iodoacetamide (IAA), and protein kinase A (bovine catalytic subunit) were obtained from Sigma-Aldrich (St. Louis, MO). Ni-NTA resin was obtained from Qiagen (Valenica, CA). Sequencing grade trypsin was purchased from Promega (Madison, WI). Micro Bio-Spin™ chromatography columns (Bio-Gel P-6 polyacrylamide gel) were obtained from Bio-Rad (Hercules, CA). PhosphoProfile™ I phosphopeptide enrichment kit containing gallium(III) chelate silica mini-spin were obtained from Sigma-Aldrich. TopTip filled with TiO_2 were purchased from Glygen (Columbia, MD). All other chemicals were HPLC grade purchased from Fisher and used without further purification.

cMyBP-C protein preparation and purification

Recombinant murine proteins muC0C2 (contains the N-terminal C0, C1, M and C2 domains of cMyBP-C) and muC1C2 (contains the C1, M and C2 domains) were cloned from the cDNA encoding the full-length murine cMyBP-C protein (GenBank gi:3747133) as described previously.[42] muC0C2 and muC1C2 were expressed in *E.coli* and purified using his-tags and Ni-NTA affinity chromatography as described previously.[43] Recombinant human C0C2 (huC0C2) and C1C2 (huC1C2) were cloned from human cDNA obtained by

RT-PCR from whole heart human total RNA (Stratagene, La Jolla, CA) using a one-step RT-PCR kit (Invitrogen, Carlsbad, CA). The resulting 2 kb PCR product was subcloned into the pCR 2.1 Topo vector (Invitrogen). PCR products encoding huC0C2 and huC1C2 were generated using gene specific primers flanking the desired domains (huC0C2 forward primer 5'-GGCCCATATGCCTGAGCCGGGGAAGAAG-3', reverse primer 5'-GGCCAAGCTTTCACTCTTTCACAAAGAGCTCCGTGCT-3'; huC1C2 forward primer 5'-GGCCCATATGGATGACCCCATTCGCCTCTTCGTC-3', reverse primer 5'-GGCCAAGCTTTCACTCTTTCACAAAGAGCTCCGTGCT-3') and cloned into the pQE-2 expression vector (Qiagen, Valencia, CA). Expression and purification of huC0C2 and huC1C2 were as described previously.[43] Protein concentrations were determined by measuring light absorbance at 280 nm (corrected for turbidity at 310 nm) and using calculated extinction coefficients from the Swiss Institute for Bioinformatics.[44]

In vitro PKA phosphorylation of C0C2 and C1C2

Expressed recombinant proteins were treated with protein kinase A (PKA, Sigma P2645) as described previously.[16, 45] Proteins (1 mg/ml) were dialyzed against a buffer containing (in mmol/L): 20 HEPES (pH 7.4), 100 KCl, 10 MgCl2, 1 ATP, and 1 DTT. PKA was resuspended in 6 mg/ml DTT and added to protein at 40 U PKA/mg protein along with an additional 1 mM ATP (2 mM final ATP concentration). The reaction was incubated at 4 °C for > 5 hours and then applied to a Ni-NTA column (Qiagen) to purify protein from PKA. Phosphorylation was confirmed by ProQ Diamond staining followed by Sypro Ruby staining (Invitrogen) according to manufacturer's instructions. The method of Ser-Asp mutation analysis of murine C1C2 protein was shown in supporting information.

In-solution tryptic digestion of proteins

The recombinant proteins (1 μg/μL) were buffer-exchanged into 50 mM ammonium bicarbonate (pH 7.8) using Micro Biospin columns. A final concentration of 5.5 mM DTT was added in 270 μL protein (100 μg) solutions to reduce disulfide bonds and incubated for 45 min at 56 °C. The resulting free thiol (-SH) groups were subsequently alkylated with IAA (10 mM final concentration) for 1 hr in the dark at room temperature. Then trypsin was added in a final enzyme:substrate ratio of 1: 50, and the digestion was carried out at 37 °C for 8 hr. The reaction was quenched by flash freezing the sample with liquid nitrogen. The tryptic digest was dried down and stored at -80 °C for further analysis.

Enrichment of tryptic phosphopeptides

Mini-spin columns containing gallium (III) chelate silica were used for the enrichment of phosphopeptides as recommended by the manufacturer with minor modifications (details in supporting information).

The phosphopeptides were also enriched using TopTips filled with TiO_2 (particle size 20~30 μm, Glygen Corp., Columbia, MD) as described in the manufacturer's instruction with minor modifications. Briefly, TiO_2 TopTips were equilibrated with 4×50 μL 0.1% TFA containing 10% ACN previously. Forty μg of the dried tryptic digest was reconstituted in 25 μL of the same solution and loaded onto TopTips with 100 repetitions assuring adequate column binding. The loaded tip was rinsed with 4×50 μL 0.1% TFA containing 10% ACN and then with 2×50 μL of MilliQ water (18 MΩ·cm) to remove unbound peptides. Finally the bound phosphopeptides were eluted sequentially with 2×50 μL of 200 mM ammonium hydroxide and 2×50 μL of 200 mM ammonium hydroxide in 10% ACN. All elution fractions were mixed, dried down and reconstituted in a solution of 2% ACN with 1% TFA just prior to nano- RPLC -MS/MS analysis.

Nano-RPLC-MS^2 and MS^3 analysis of cMyBP-C tryptic digest

Nano-RPLC tandem mass spectrometric analysis was performed on an LTQ-OrbitrapXL mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with an ADVANCE nanospray ion source (Michrom Bioresources, Auburn, CA), a Surveyor MS pump (ThermoFisher), and a microautosampler (ThermoFisher). The dried tryptic peptide mixture was dissolved in 2% ACN with 1% TFA and separated on a 100-μm-ID IntegraFrit™ column (New Objective, Woburn, MA) packed in house with reversed-phase C_{18} material. The column was packed to a length of 18 cm with a 100% methanol slurry of Magic C_{18}AQ material (5 μm particle size, 200-Å pore size, Michrom Bioresources) using a high pressure cell pressurized with helium. The column was equilibrated before sample injection. Then the sample was loaded for 20 min at 2% solvent B (0.1% (v/v) formic acid in ACN) and 98% solvent A (0.1% (v/v) formic acid in water) at a flow rate of 250 nL/min. Peptides were resolved by the following gradient at a flow rate of 650 nL/min: 2-10% solvent B in 10 min, 10-35% solvent B in 70 min, 35-70% solvent B in 5 min, then increased to 90% solvent B in 3 min, maintained at 90% for 10 min, and then reversed to 2% buffer B. The LTQ-OrbitrapXL mass spectrometer was operated in the data-dependent acquisition mode to automatically switch between Orbitrap-MS, Orbitrap-MS^2 and Orbitrap-MS^3 acquisition. Survey scans were acquired in profile mode and MS^2 with MS^3 scans were acquired in centroid mode. TOP5 and the neutral loss/MS^3 method was used to acquire MS^2 and MS^3 data (see supporting information).

Peptide identification via MASCOT database search

MASCOT software (version 1.9.05, Matrix Science, London, U.K.) was used for database searching. MASCOT DTA format files were converted from the RAW data files acquired for each LC-MS run using BioworksBrowser software with default parameters (version 3.3.1 SP1, ThermoFisher Scientific). The resulting Sequest (.DTA) files from each run were merged into one single Mascot Generic Format (MGF) file via merge.pl (version 2007.3.9, Matrix Science, downloaded from http://www.matrixscience.com/downloads/merge.zip) and searched against an in-house database (584 protein entries, contained murine and human C1C2, C0C2 sequences, and their reversed sequences, detailed in Supporting Information; the database also contained frequently occurring contaminants including human keratins, porcine trypsin, bovin serum albumin, bovin beta-casein, bovin PKA and their reversed sequences). Searches were performed with tryptic specificity allowing four missed cleavages and a tolerance on the mass measurement of 25 ppm in MS mode and 0.1 Da for MS^2 ions. The instrument setting was specified as “ESI-Trap”. Possible structure modifications allowed were protein N-terminal acetylation, carbamidomethylation of Cys; oxidation of Met and phosphorylation of Ser, Thr, and Tyr. All identified peptides have an individual MS^2 ion score greater than 18, corresponding to a statistically significant (p value<0.05) confident identification which was set as default by MASCOT algorithm (More details in supporting information).

Mass spectrometry analysis of intact C1C2/C0C2

A nanospray LCT Premier bench top orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer (Waters, Manchester, U.K.) was used to collect mass spectra of the intact muC1C2, muC1C2P and huC1C2 proteins. The analysis was carried out in positive V mode in which the ions are reflected through a reflectron to obtain an instrument resolution of approximately 6,000. Proteins (1 μg/μL) were buffer exchanged into 50 mM ammonium acetate (pH 7.5) and 1 mM DTT using Micro Bio-spin P6 columns (Bio-Rad, Hercules, CA), and 5 μL aliquots were introduced via nanoflow capillaries prepared in house. The capillary and cone voltages were set at 1,200 V and 50 V, respectively. The desolvation and source temperatures were set at 200 °C and 50 °C, respectively. All the other voltages were optimized to provide maximal signal intensity in each of the modes. All raw data were

processed and measured using Waters MassLynx (Version 4.1). Waters Masslynx MaxEnt 1 software was used to obtain the deconvoluted peak.

A nanospray SYNAPT HDMS hybrid quadrupole ion mobility oa-TOF mass spectrometer (Waters, Manchester, U.K.) was operated in normal Q-TOF mode and used to collect mass spectra of the intact huC1C2P, muC0C2, muC0C2P, huC0C2, and huC0C2P (details in supporting information).

Results and Discussion

In vitro PKA phosphorylation of murine and human C1C2 and C0C2

Both murine and human recombinant proteins C0C2 (contains the four N-terminal C0, C1, M and C2 domains of cMyBP-C (Fig. S1A)), and C1C2 (contains C1, M and C2 domains) were treated with PKA to phosphorylate residues within the M-domain.[16, 45] SDS-Page gel analysis with ProQ Diamond staining of proteins before and after PKA treatment confirmed protein phosphorylation (Figure 1). MuC1C2, muC0C2, huC1C2, and huC0C2 do not stain with ProQ Diamond prior to treatment with PKA indicating that these proteins were not phosphorylated following expression in bacteria. However, after treatment with PKA, muC1C2P, muC0C2P, huC1C2P, and huC0C2P phosphorylation was evident as indicated by ProQ Diamond staining.

Nano-LC-MS/MS and MS^3 analysis of the murine cMyBP-C tryptic digest

The recombinant proteins muC1C2, muC1C2P, muC0C2 and muC0C2P were reduced, alkylated, and digested successively. The resulting murine C1C2/C1C2P/C0C2/C0C2P tryptic peptides were subsequently injected into a nano-RPLC column coupled to a LTQ-Orbitrap mass spectrometer respectively, and both MS^2 and MS^3 analysis were carried out using data-dependent acquisition mode as described under “Experimental Procedures”. The neutral loss-dependent MS^3 mode was used in addition to the data-dependent TOP5 LC-MS/MS acquisition for the correct identification of phosphopeptides and corresponding phosphorylation sites in the cMyBP-C proteins. This strategy has been shown to precisely identify PTMs of proteins.[32, 46-48] Results show all 4 murine cMyBP-C proteins (muC1C2, muC1C2P, muC0C2 and muC0C2P) were unambiguously identified with better than 85% sequence coverage (Table I). No phosphorylation sites were found in muC1C2 and muC0C2 prior to treatment with PKA. In contrast, three phosphorylation sites (Ser^{282}, Ser^{302} and Ser^{307}) were found in muC1C2P and muC0C2P and a fourth phosphorylation site Ser^{273} was found in muC1C2P and muC0C2P after selective enrichment of phosphopeptides by TiO_2 and Ga(III) IMAC (Table II). These results are in agreement with the ProQ Diamond staining results (Fig. 1). There are a total 49 potential phosphorylation sites (27 Ser, 15 Thr and 7 Tyr) and 75 potential phosphorylation sites (43 Ser, 23 Thr and 9 Tyr) in muC1C2 and muC0C2, respectively (Supplemental Figures S2 and S3). However, only four serines were found to be phosphorylated by PKA *in vitro*, and all four sites are located in the M-domain located between the C1 and C2 domains in the N-terminus of cMyBP-C. This is the first report of the identification of four PKA phosphorylation sites (Ser^{273}, Ser^{282}, Ser^{302} and Ser^{307}) in murine cMyBP-C, with Ser^{307} being a novel site.

Figure 2. shows the identification of $Ser(P)^{307}$ from muC1C2P using MS/MS and neutral loss/MS^3 method. At an elution time of 78.81 min (Fig. 2A), a precursor ion was observed in the Orbitrap survey scan (Fig. 2B) with a 2+ charge state and a monoisotopic mass at *m/z* of 1118.5392 (Δm= 2.9 ppm). This ion was automatically isolated in the linear ion trap and fragmented by CID to produce the MS/MS spectrum (Fig. 2C) from which the first most intense ion corresponding to the neutral loss precursor ion (*m/z* = 1069.55) was automatically selected and fragmented to yield an MS^3 spectrum (Fig. 2D). All of the

precursor ions and fragment ions in MS/MS and MS^3 were recorded by the Orbitrap. The tryptic phosphopeptide (amino acids 305–322, murine sequence numbering) was unambiguously identified by the MASCOT search with a top score of 65 (where a score greater than 18 indicates identity corresponding to a statistically significant ($p<0.05$) confident identification in MASCOT), and Ser^{307} was identified as being phosphorylated based on the b and y fragment ions present in the MS/MS spectrum. The precursor ion $[M+2H]^{2+}$ *m/z* 1118.5392 indicates that there is one phosphorylation site in this peptide (RDpSKLEAPAEEDVWEILR), since the $[M+2H]^{2+}$ of non-phosphopeptide RDSKLEAPAEEDVWEILR is *m/z* 1078.5527. Subsequent MS^2 analysis showed the neutral loss of H_3PO_4 (98 Da) occurred on both the b_3 ion (RDpS) and the y_{16} ion (pSKLEAPAEEDVWEILR) (Fig. 2C), and this potential site of phosphorylation was also supported by MS^3. The phosphoserine is converted to a dehydroalanine residue during CID, and a predominant series of b and y fragment ions are present in the MS^3 spectrum (Fig. 2D). Ser^{307} in murine cMyBP-C is a novel PKA phosphorylation site that has not been reported previously.

Nano-LC-MS/MS and MS^3 analysis of the human cMyBP-C tryptic digest

In human cMyBP-C, three PKA phosphorylation sites have previously been reported (Ser^{275}, Ser^{284}, and Ser^{304}) [16] using the approach of mass measurements of intact proteins. Ser^{273}, Ser^{282}, and Ser^{302} are the three homologous sites that exist in the murine isoform which were identified in the present study. Because Ser^{307} in murine cMyBP-C is also homologous to human cMyBP-C (Ser^{311}), we chose to investigate if Ser^{311} is phosphorylated by PKA *in vitro*. Mass spectrometry analysis revealed a sequence coverage of 98% for the huC1C2, 99% for huC1C2P, 95% for huC0C2, and 96% for huC0C2P (see Table I and the online supplemental materials). Four PKA phosphorylation sites were identified: Ser^{275}, Ser^{284}, and Ser^{304}, and Ser^{311} (Table I, II and supplemental material), with Ser^{311} being a novel site (Fig. 3). At an elution time of 80.10 min (Fig. 3A), a precursor ion was observed in the Orbitrap survey scan (Fig. 3B) with a 3+ charge state and a monoisotopic mass at *m/z* of 812.0599 (Δm = 0.1 ppm). This ion was isolated in the linear ion trap and fragmented by CID to produce the MS/MS spectrum (Fig. 3C) from which the first most intense ion corresponding to the neutral loss precursor ion (*m/z* = 779.40) was selected and fragmented to yield an MS^3 spectrum (Fig. 3D). The tryptic phosphopeptide (amino acids 307-326, human sequence numbering) was reported with a MASCOT score of 51, and Ser^{311} was identified as being phosphorylated based on the b and y fragment ions present in the MS/MS spectrum. Subsequent MS^2 analysis showed neutral loss of H_3PO_4 (98 Da) occurring on the b_5 ion (TPRDpS) and y_{16} ion (pSKLEAPAEEDVWEILR) illustrating Ser^5 as phosphorylated (Fig. 3C). Additional MS^3 of *m/z* 779.40 validated the phosphorylation site and peptide sequence.

$Ser(P)^{311}$ was not detected in a previous study of human cMyBP-C M-domain PKA phosphorylation.[16] One possible reason that the previous study did not observe phosphorylated Ser^{311} may be due to the unstable state of this phosphorylation site and/or sub-stoichiometric amounts of the phosphopeptides containing the $Ser(P)^{311}$ site. In this study, we found both phosphorylated Ser^{311} and un-phosphorylated Ser^{311} in the same nano-LC-MS/MS run of huC1C2P and huC0C2P. Other identified phosphorylation sites were not found in the un-phosphorylated state. Figure S10 shows the identification of Ser^{311} (elution time 82.18 min) and $Ser(P)^{311}$ (89.12 min) from huC1C2P using the nano-LC-MS/MS with neutral loss/MS^3 method. Both the un-phosphorylated peptide and phosphopeptide were observed in the same LC-MS/MS analysis of raw protein digests suggesting that this phosphorylation site is unstable and/or in low abundance.

Mass Spectrometric analysis of the intact C1C2/C0C2 proteins

To validate the identified phosphorylation sites in murine and human cMyBP-C recombinant proteins, the accurate mass of the intact (i.e., undigested) proteins were measured by ESI-TOF mass spectrometry. The employment of LCT Premier bench-top oa-TOF allowed observation of the intact muC1C2 and muC1C2P protein as shown in Fig. 4. The measured molecular mass of the muC1C2 is 34635.1±2.9 Da (Fig. 4A). The mass difference between measured and theoretical value is 1.2 Da (35 ppm) which indicates that there are no PTMs in muC1C2 protein. The measured molecular mass of the muC1C2P is 34953.6±0.5 Da (Fig. 4B). Good correlation (2.6 Da, 74 ppm) between experimental mass and the theoretical mass (34956.2 Da) of muC1C2P indicates the presence of four phosphorylation sites in this protein. Given the data from the mass measurement analysis of intact muC1C2 and muC1C2P protein, we can validate that muC1C2P is tetra-phosphorylated. More mass measurements analysis about intact muC0C2/muC0C2P, huC1C2/huC1C2P and huC0C2/huC0C2P were also confirmed our discovery from bottom-up approach (Figures S11-13).

The finding of a fourth PKA site in the mouse cMyBP-C M-domain (Ser^{307}) was anticipated because a homologous site was recently identified in the rat and the dog (note exact sequence homology, Figure S1). However, the presence of a fourth PKA site in the M-domain of the human isoform (Ser^{311}) was more of a surprise, as previously only three PKA sites had been identified in the human isoform[16, 17] and the five amino acids leading up to and including Ser^{311} (^{307}TPRDS^{311}) does not constitute a usual PKA phosphorylation site motif.[49] Previous studies used recombinant huC1C2 and identified three sites through site-directed mutagenesis and mass spectrometry analysis of intact proteins.[16, 17] In this study, we found that the intensities of the intact protein peaks that correspond to huC0C2P and huC1C2P containing four phosphate groups were smaller than the intensities of peaks corresponding to huC0C2P and huC1C2P containing three phosphate groups (Figures S12 and S13). Due to this and the variable phosphorylation indicated by the bottom-up results, it is likely that $Ser(P)^{311}$ was not detected in previous studies.

Confirmation that Ser^{307} is phosphorylated in muC1C2 using site-directed mutagenesis

Mutant muC1C2 proteins were created that contained substitutions of phosphorylatable serine residues to aspartic acid residues in order to block phosphorylation by PKA and thus determine specific phosphorylation sites. As shown in Fig. 5, after treatment with PKA muC1C2 stains with ProQ Diamond, indicating phosphorylation. The mutant muC1C2(3S-D) (containing S273D, S282D, and S302D mutations) was created to block PKA phosphorylation at the first three PKA phosphorylation sites, but not the novel Ser^{307} site determined in the mass spectrometric analyses. After treatment with PKA, muC1C2(3S-D) stained with ProQ Diamond (Fig. 5), indicating PKA phosphorylation at additional serine residues, likely at Ser^{307}. The mutant muC1C2(4S-D) (containing the mutations S273D, S282D, S302D, and S307D) was next created to block PKA phosphorylation at all four PKA phosphorylation sites. Treatment of muC1C2(4S-D) with PKA did not further phosphorylate the mutant protein (Fig. 5), demonstrating that Ser^{307} is phosphorylated *in vitro* by PKA.

Conclusion

The goal of this study was to assess the phosphorylation state of the human and murine cMyBP-C motif, or M-domains using mass spectrometry. These results mark the first time that complete mass spectrometric analyses (bottom-up approach and accurate mass measurement of intact proteins) were used to assess the phosphorylation state of the N-terminus of murine cMyBP-C. The significant findings of this study were the identification of four PKA phosphorylation sites in both the human and murine isoforms, with the characterization of novel sites (Ser^{311} and Ser^{307} in the human and murine isoforms,

respectively). These results provide further evidence towards the complete characterization of the phosphorylation state of cMyBP-C.

The incorporation of complementary bottom-up mass spectrometry approach and mass measurements of intact proteins method were used to characterize the phosphorylation state of murine and human cMyBP-C recombinant proteins after PKA treatment *in vitro*. Although the accurate measurements of intact proteins readily provide excellent intact protein masses which indicate post-translational modifications, it can miss some PTMs due to sub-stoichiometric amounts and/or dynamic time duration. And moreover, it may represent heterogeneous populations of the proteins which mean the underlying proteins may feature the same number of PTMs but at different sites. In contrast, the bottom-up method can provide good sequence coverage and precise locations of PTM sites. Combining the bottom-up and accurate mass measurements of intact proteins provides both maximum coverage and precise PTM identification. In this study, all of the proteins obtained higher than 85% sequence coverage. Ser^{273}, Ser^{282}, Ser^{302} and Ser^{307} in murine isoforms and Ser^{275}, Ser^{284}, Ser^{304} and Ser^{311} in human isoforms can be phosphorylated by PKA *in vitro*. The biochemical mutation analysis (Ser to Asp) also demonstrated this discovery in murine isoform proteins. But whether the novel discovered phosphorylation sites will carry physiological significance is still unclear; future studies are needed to verify usage of these phosphorylation sites in murine and human cMyBP-C following PKA stimulation *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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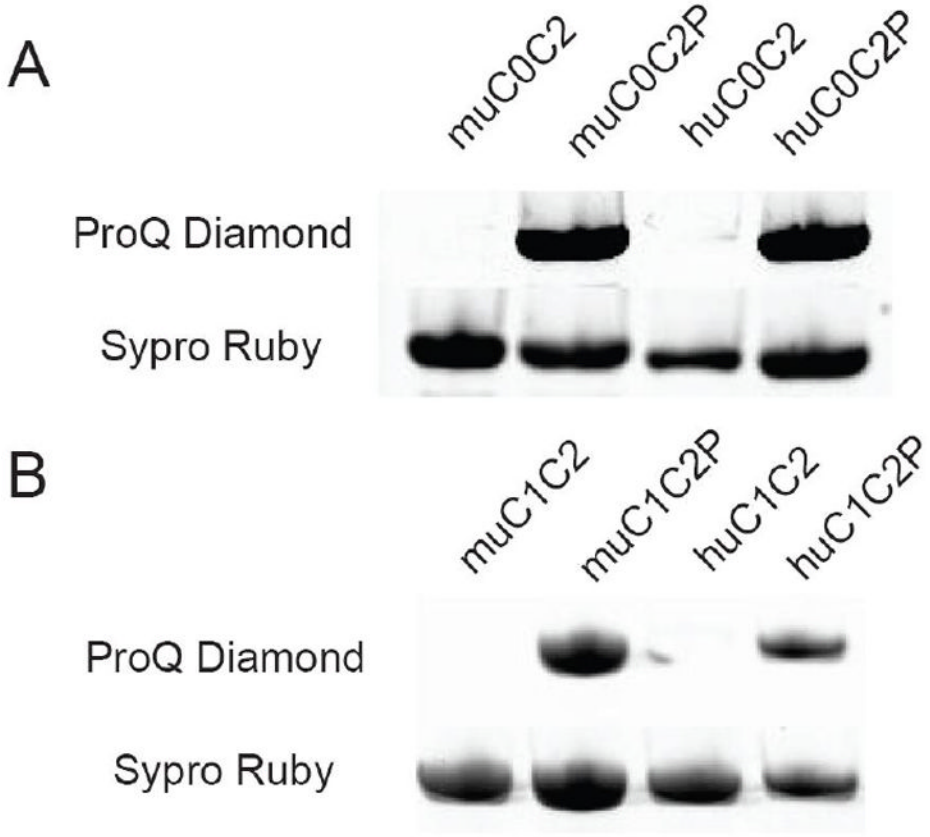

Figure 1.
PKA phosphorylation of recombinant proteins detected by SDS-PAGE with ProQ Diamond staining. (A) Phosphorylation of muC0C2P and huC0C2P is indicated by ProQ Diamond staining, whereas muC0C2 and huC0C2 were not stained prior to treatment of PKA. Sypro Ruby stains for total protein. (B) muC1C2P and huC1C2P stain with ProQ Diamond, but muC1C2 and huC1C2 do not.

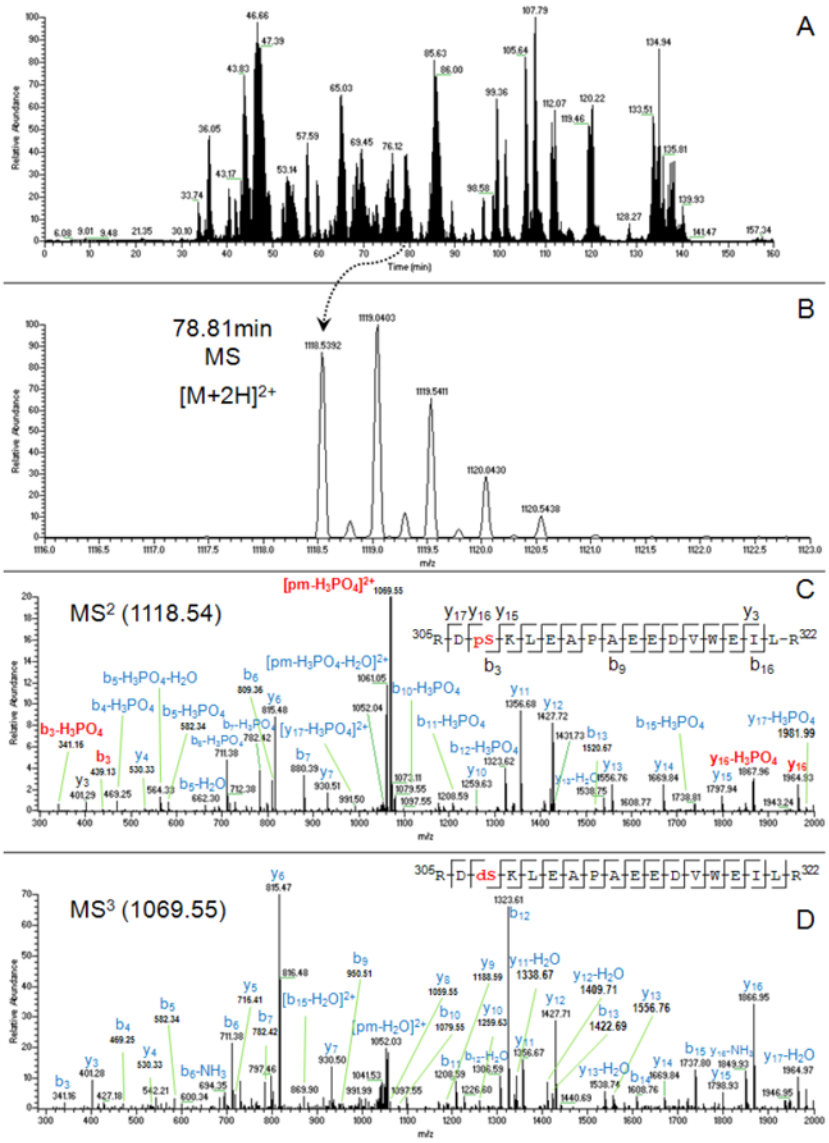

Figure 2.

Identification of the phosphorylation site Ser(P)^{307} from muC1C2P using neutral loss/MS^3 method. A, base peak chromatogram. B, precursor mass scan at 78.81 min using Orbitrap. For illustration purposes, only the 1116.0-1123.0 *m/z* mass region of the [M+ 2H]$^{2+}$ ion is shown. C, MS^2 scan of the *m/z* 1118.54 ion. D, MS^3 scan of the *m/z* 1069.55. The peptide sequence identified by MASCOT and validated by manual confirmation from the raw MS^2 data is shown in the *inset* of C. The peptide sequence validated by manual confirmation from the raw MS^3 data is shown in the inset of D where *dS* represents the dehydroalanine residue. *pm*, precursor ion; *pS*, phosphoserine.

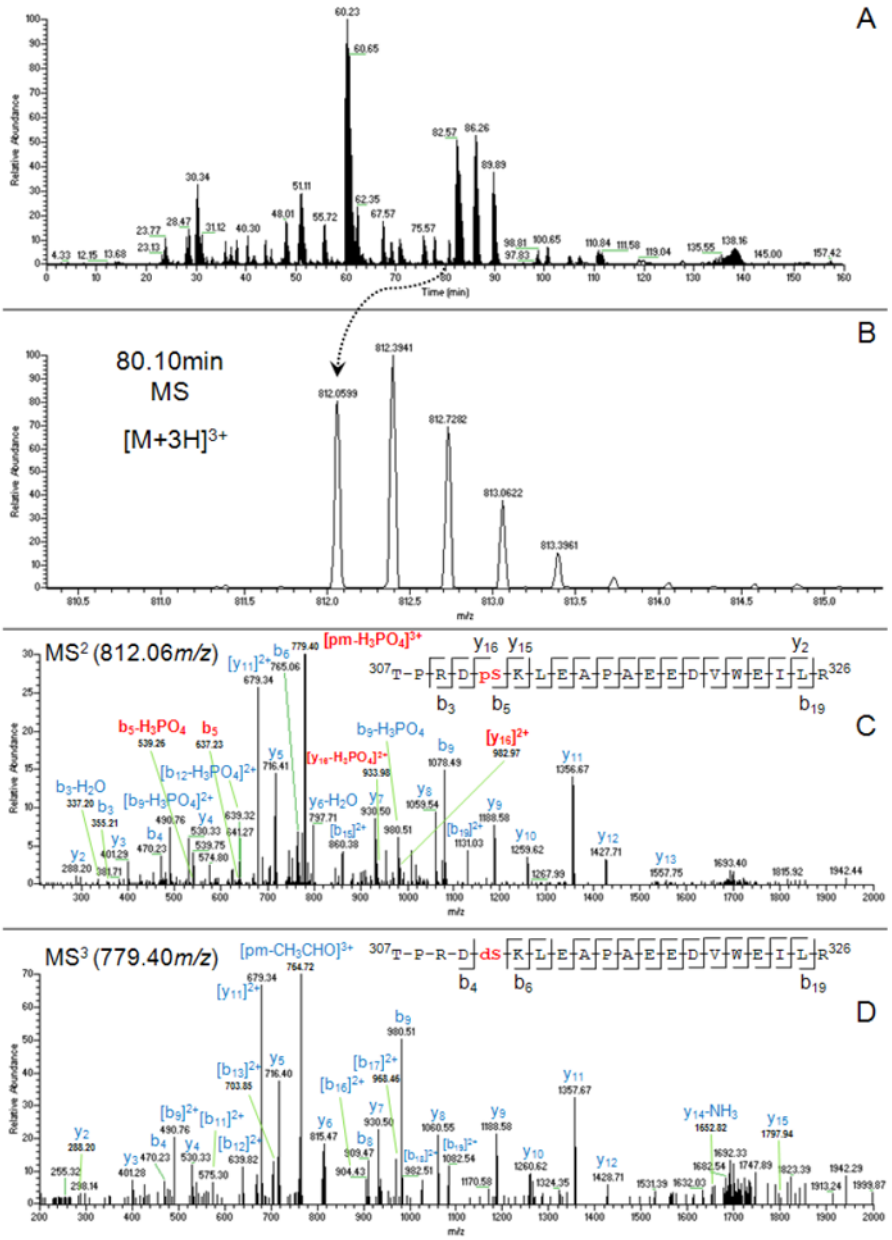

Figure 3.

Identification of the phosphorylation site Ser(P)^{311} from huC1C2P using neutral loss/MS^3 method. A, base peak chromatogram. B, precursor mass scan at 80.10 min using Orbitrap. For illustration purposes, only the 810.3-815.3 *m/z* mass region of the [M+ 3H]$^{3+}$ ion is shown. C, MS^2 scan of the *m/z* 812.06 ion. D, MS^3 scan of the *m/z* 779.40. The peptide sequence identified by MASCOT and validated by manual confirmation from the raw MS^2 data is shown in the inset of C. The peptide sequence validated by manual confirmation from the raw MS^3 data is shown in the *inset* of D. *pm*, precursor ion; *pS*, phosphoserine; *dS*, dehydroalanine residue.

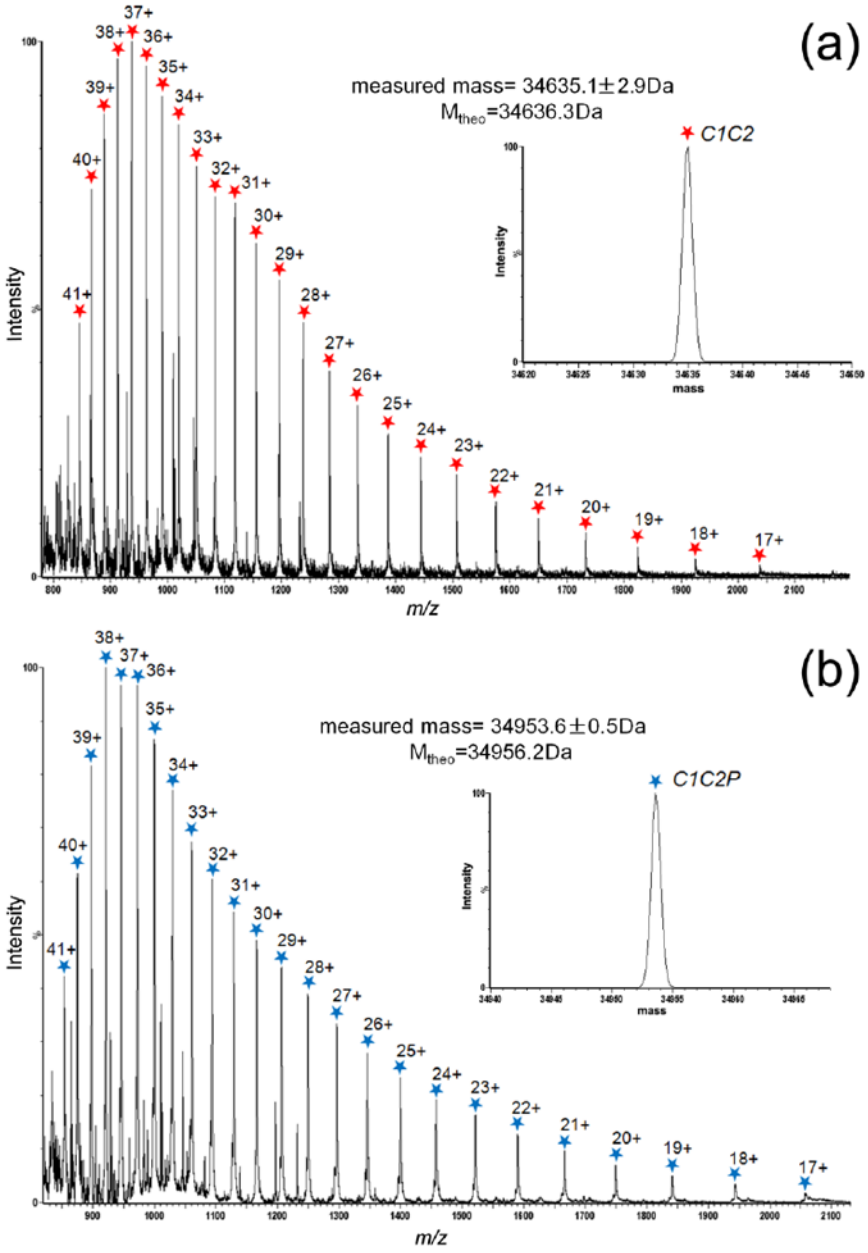

Figure 4.

Mass spectrometric analysis of the intact muC1C2 protein and muC1C2P protein. (a) Identification of muC1C2 protein. (b) Identification of muC1C2P protein. The inset spectra represent the deconvoluted mass of muC1C2 and muC1C2P respectively, which calculated by Waters Masslynx MaxEnt 1 software. The theoretical average mass of the protein was calculated by Waters MassLynx. The corresponding charge states are indicated on each peak in the raw data.

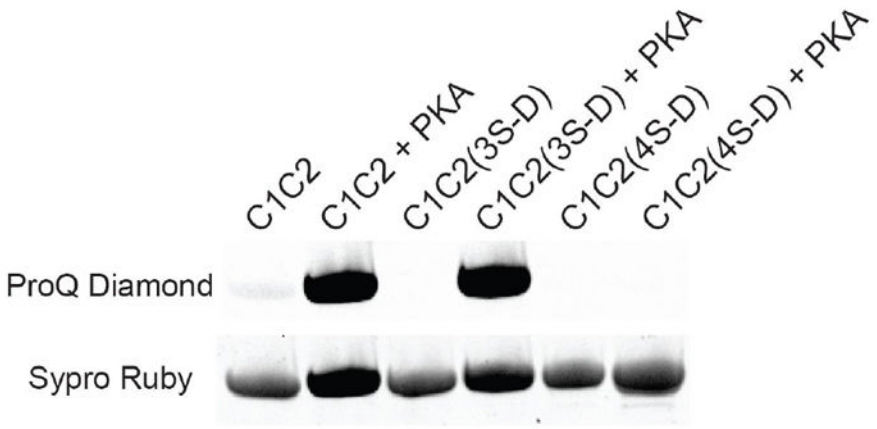

Figure 5.

Mutation analysis shows that Ser^{307} is phosphorylated in muC1C2. Recombinant muC1C2 proteins were treated with PKA and analyzed using SDS-PAGE with ProQ Diamond staining (phosphoprotein) and Sypro Ruby (total protein). C1C2(3S-D) contains the mutations S273D, S282D, and S302D. When treated with PKA, C1C2(3S-D) stains with ProQ Diamond, indicating additional phosphorylation by PKA. C1C2(4S-D) contains the mutations S273D, S282D, S302D, and S307D. PKA treatment of C1C2(4S-D) did not result in ProQ Diamond staining, indicating that phosphorylation is completely blocked by the aspartic acid substitutions, and confirming that Ser^{307} is phosphorylated by PKA in muC1C2.

Table 1

Identification of reconbinant cMyBP-C fragment proteins and their corresponding PTMs using nano-LC-LTQ-Orbitrap

Protein Name	Molecular Mass (Da)[a]	Amino Acids Number	pI[b]	Unique Peptides Detected[c]	Sequence Coverage	Post-translational modifications
muC1C2	34636.2	309	8.18	55	93%	None
muC1C2P	34956.2	309	6.42	44	89%	Ser (P)^{273}, Ser (P)^{282}, Ser (P)^{302}, Ser (P)^{307}
muC0C2	49873.0	456	6.49	41	85%	None
muC0C2P	50192.9	456	6.01	42	93%	Ser (P)^{273}, Ser (P)^{282}, Ser (P)^{302}, Ser (P)^{307}
huC1C2	35046.9	311	8.18	44	98%	None
huC1C2P	35366.8	311	6.42	62	99%	Ser (P)^{275}, Ser (P)^{284}, Ser (P)^{304}, Ser (P)^{311}
huC0C2	49956.6	460	8.12	52	95%	None
huC0C2P	50276.5	460	6.46	57	96%	Ser (P)^{275}, Ser (P)^{284}, Ser (P)^{304}, Ser (P)^{311}

[a] Calculated from the theoretical average mass of the corresponding cMyBP-C fragment proteins plus any post-translational modifications identified.

[b] Theoretical pI of each intact protein in its unmodified form was calculated using ProtParam (http://www.expasy.ch/tools/protparam.html). The pI of proteins in the phosphorylated form were calculated using p*I*Calculator (version 1.0, downloaded from https://bioinformatics.chem.uu.nl/supplementary/gauci_proteomics/ as an executable java JAR. Use default pKa set in this software according to EXPASY and ProMoST) when the total number of dectected phosphorylation sites were provided.

[c] Only the peptides with MASCOT individual ions scores more than threshold score 18 were accepted (p value<0.05). Covalently modified peptides count as unique; different charge states or multiple fragmentation spectra of the same peptide are excluded.

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Table 2

Phosphopeptides and corresponding phosphorylation sites identified in cMyBP-C fragments tryptic digest using nano-LC-LTQ-Orbitrap

Phosphorylation Site(s) Identified[a]	Protein Source	Peptide Start-end[a]	Sequence	no. of PO_4	Mr (Da)	Charge State & (MASCOT score [b])	LC-MS/MS NL[c]/MS^3	Without Enrichment	Enrichment	
									TiO_2	IMAC
Ser $(P)^{273}$	muC1C2P&muC0C2P	271-279	RTpSLAGAGR	1	967.4600	2+(47)	Yes	No	Yes	Yes
	muC1C2P&muC0C2P	272-279	TpSLAGAGR	1	811.3589	2+(26)	Yes	No	Yes	Yes
Ser $(P)^{282}$	muC1C2P&muC0C2P	280-298	RTpSDSHEDAGTLDFSSLLK	1	2157.9579	2+(58), 3+(33)	Yes	Yes	Yes	Yes
	muC1C2P&muC0C2P	280-299	RTpSDSHEDAGTLDFSSLLKK	1	2286.0529	3+(20)	Yes	Yes	No	No
	muC1C2P&muC0C2P	281-298	TpSDSHEDAGTLDFSSLLK	1	2001.8568	2+(50), 3+(24)	Yes	Yes	Yes	Yes
Ser $(P)^{302}$	muC1C2P&muC0C2P	299-304	KRDpSFR	1	887.4014	2+(23), 3+(21)	Yes	Yes	Yes	Yes
	muC1C2P	300-304	RDpSFR	1	759.3065	2+(25)	Yes	Yes	No	No
	muC1C2P	300-305	RDpSFRR	1	915.4076	2+(23)	Yes	Yes	No	Yes
	muC1C2P	301-304	DpSFR	1	603.2054	2+(21)	Yes	No	Yes	No
	muC0C2P	299-305	KRDpSFRR	1	1043.5025	2+(20)	Yes	No	Yes	Yes
Ser $(P)^{302}$&Ser $(P)^{307}$	muC1C2P	300-322	RDpSFRRDpSKLEAPAEEDVWEILR	2	2976.3531	3+(21), 4+(32)	Yes	Yes	Yes	Yes
	muC1C2P	301-322	DpSFRRDpSKLEAPAEEDVWEILR	2	2820.2520	3+(49)	Yes	Yes	Yes	Yes
	muC0C2P	299-322	KRDpSFRRDpSKLEAPAEEDVWEILR	2	3104.4481	4+(22)	Yes	Yes	Yes	Yes
Ser $(P)^{307}$	muC1C2P&muC0C2P	305-322	RDpSKLEAPAEEDVWEILR	1	2235.0572	2+(65), 3+(45)	Yes	Yes	Yes	Yes
	muC1C2P&muC0C2P	306-322	DpSKLEAPAEEDVWEILR	1	2078.9561	2+(49), 3+(24)	Yes	Yes	Yes	Yes
Ser $(P)^{275}$	huC1C2P&huC0C2P	273-281	RTpSLAGGGR	1	953.4443	2+(47)	Yes	Yes	Yes	Yes
	huC1C2P	273-282	RTpSLAGGGRR	1	1109.5454	2+(21)	Yes	NO	Yes	Yes
	huC1C2P	274-281	TpSLAGGGR	1	797.3432	2+(27)	Yes	NO	Yes	Yes
Ser $(P)^{275}$&Ser $(P)^{284}$	huC1C2P	273-300	RTpSLAGGGRRIpSDSHEDTGILDFSSLLK	2	3147.4750	3+(44), 4+(40)	Yes	Yes	Yes	Yes
	huC1C2P	273-301	RTpSLAGGGRRIpSDSHEDTGILDFSSLLKK	2	3275.5700	4+(25)	Yes	Yes	Yes	Yes
	huC1C2P	274-300	TpSLAGGGRRIpSDSHEDTGILDFSSLLK	2	2991.3739	4+(26)	Yes	Yes	Yes	Yes
Ser $(P)^{284}$	huC1C2P&huC0C2P	282-300	RIpSDSHEDTGILDFSSLLK	1	2212.0412	2+(53), 3+(38)	Yes	Yes	Yes	Yes
	huC1C2P &huC0C2P	282-301	RIpSDSHEDTGILDFSSLLKK	1	2340.1362	2+(21), 3+(49)	Yes	Yes	Yes	Yes
	huC1C2P&huC0C2P	283-300	IpSDSHEDTGILDFSSLLK	1	2055.9401	2+(64), 3+(29)	Yes	Yes	Yes	Yes
	huC1C2P	283-301	IpSDSHEDTGILDFSSLLKK	1	2184.0351	2+(43)	Yes	Yes	Yes	Yes
Ser $(P)^{304}$	huC1C2P	301-309	KRDpSFRTPR	1	1241.6030	2+(20)	Yes	No	Yes	Yes
	huC1C2P	302-309	RDpSFRTPR	1	1113.5080	2+(30)	Yes	No	Yes	Yes

Phosphorylation Site(s) Identified[a]	Protein Source	Peptide Start-end[a]	Sequence	no. of PO_4	Mr (Da)	Charge State & (MASCOT score [b])	LC-MS/MS NL[c]/MS^3	Without Enrichment	Enrichment	
									TiO_2	IMAC
Ser $(P)^{311}$	huC1C2P	303-309	DpSFRTPR	1	957.4069	2+(22)	Yes	No	Yes	Yes
	huC0C2P	301-306	KRDpSFR	1	887.4014	2+(21)	Yes	No	Yes	Yes
	huC1C2P&huC0C2P	307-326	TPRDpSKLEAPAEEDVWEILR	1	2433.1576	3+(51)	Yes	Yes	Yes	Yes
	huC1C2P&huC0C2P	310-326	DpSKLEAPAEEDVWEILR	1	2078.9561	2+(54)	Yes	Yes	Yes	No

[a] All sites locations refer to their natural positions *in vivo*.

[b] The threshold score for accepting individual MS/MS spectra is 18, corresponding to a statistically significant (p value<5%) confident identification in MASCOT. All identified phosphopeptides were validated by manual confirmation from raw MS^2 and MS^3 data.

[c] NL, neutral loss of H_3PO_4.