A Critical Function for Ser-282 in Cardiac Myosin Binding Protein-C Phosphorylation and Cardiac Function

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Abstract: Cardiac myosin-binding protein-C (cMyBP-C) phosphorylation at Ser-273, Ser-282, and Ser-302 regulates myocardial contractility. In vitro and in vivo experiments suggest the nonequivalence of these sites and the potential importance of Ser-282 phosphorylation in modulating the protein’s overall phosphorylation and myocardial function. The objective was to determine whether complete cMyBP-C phosphorylation is dependent on Ser-282 phosphorylation and to define its role in myocardial function. We hypothesized that Ser-282 regulates Ser-302 phosphorylation and cardiac function during β-adrenergic stimulation. Using recombinant human C1-mol/L-C2 peptides in vitro, we determined that protein kinase A can phosphorylate Ser-273, Ser-282, and Ser-302. Protein kinase C can also phosphorylate Ser-273 and Ser-302. In contrast, Ca2+-calmodulin-activated kinase II targets Ser-302 but can also target Ser-282 at nonphysiological calcium concentrations. Strikingly, Ser-302 phosphorylation by Ca2+-calmodulin-activated kinase II was abolished by ablating the ability of Ser-282 to be phosphorylated via alanine substitution. To determine the functional roles of the sites in vivo, three transgenic lines, which expressed cMyBP-C containing either Ser-273-Ala-282-Ser-302 (cMyBP-C\(^{\text{SAS}}\)), Ala-273-Asp-282-Ala-302 (cMyBP-C\(^{\text{ADA}}\)), or Asp-273-Ala-282-Asp-302 (cMyBP-C\(^{\text{DAD}}\)), were generated. Mutant protein was completely substituted for endogenous cMyBP-C by breeding each mouse line into a cMyBP-C null (t/t) background. Serine-to-alanine substitutions were used to ablate the abilities of the residues to be phosphorylated, whereas serine-to-aspartate substitutions were used to mimic the charged state conferred by phosphorylation. Compared to control nontransgenic mice, as well as transgenic mice expressing wild-type cMyBP-C, the transgenic cMyBP-C\(^{\text{SAS}}\), cMyBP-C\(^{\text{ADA}}\), and cMyBP-C\(^{\text{DAD}}\) mice showed no increases in morbidity and mortality and partially rescued the cMyBP-C\(^{\text{A}}\) phenotype. The loss of cMyBP-C phosphorylation at Ser-282 led to an altered β-adrenergic response. In vivo hemodynamic studies revealed that contractility was unaffected but that cMyBP-C\(^{\text{SAS}}\) hearts showed decreased diastolic function at baseline. However, the normal increases in cardiac function (increased contractility/relaxation) as a result of infusion of β-agonist was significantly decreased in all of the mutants, suggesting that competency for phosphorylation at multiple sites in cMyBP-C is a prerequisite for normal β-adrenergic responsiveness. Ser-282 has a unique regulatory role in that its phosphorylation is critical for the subsequent phosphorylation of Ser-302. However, each residue plays a role in regulating the contractile response to β-agonist stimulation.

Key Words: contractile function ■ myofilament ■ myosin-binding protein-C ■ phosphorylation

Cardiovascular disease, particularly ischemia, myocardial infarction, and heart failure, constitutes a growing health and economic problem, afflicting approximately five million people in the United States each year at an estimated cost of $29.6 billion.1 Heart failure is associated with diminished β-adrenergic (β-AR) receptor responsiveness, loss of cardiac contractility, abnormalities in Ca2+ handling,2,3 and altered contractile protein phosphorylation.4 Altered phosphorylation of the contractile proteins may partially underlie cardiac dysfunction in heart failure.4,5 Cardiac myosin-binding protein-C (cMyBP-C) phosphorylation at multiple sites can impact myocardial function,6,7 metabolism,8 and cardiop...
The critical role of Ser-282 phosphorylation in the cardiac sarcomere. It also binds to titin via domains C8 to C10 and presumably to actin as well; however, this interaction is less well-defined, suggesting that these interactions may be necessary for the stability or function of cMyBP-C in the sarcomeres.

The cardiac isoform differs from the skeletal isoform in that it contains an extra domain at the N-terminus (C0) and a phosphorylation motif (M-domain) localized between the immunoglobulin I-like C1 and C2 domains, in which Ser-273, Ser-282, and Ser-302 each can be phosphorylated. The three sites are differentially phosphorylated by the activated kinase II (CaMKII), PKD, and the 90-kDa ribosomal S6 kinase. To begin to explore the physiological roles of cMyBP-C phosphorylation, we previously established two transgenic (TG) mouse models in which the three phosphorylation sites were mutated to either alanine or aspartate (phospho-mimetic, AllP). Our findings suggested that cMyBP-C phosphorylation is essential for normal cardiac function and that charged residues at 273, 282, and 302 sites conferred cardiomyopathy.

Materials and Methods

Evaluation of cMyBP-C Phosphorylation

To define the phosphorylation status of Ser-273, Ser-282, and Ser-302, myofibrils from nontransgenic (NTG) and TG mouse hearts were treated with either PKA, PKC, CaMKII, or phosphatase (Sigma), electrophoresed on SDS-PAGE, and Western blots were performed using an anti-cMyBP-C2-14 antibody that was raised against the residues participates in the contractile response to β-AR stimulation in vivo.

Cardiac Function

Cardiac function was determined in the intact closed chest model as described. Increasing doses of dobutamine were administered during 3-minute constant infusions (0.1 μL/min/g body weight) to determine β-AR responsiveness. To observe changes in left ventricular chamber size and fractional shortening, noninvasive M-mode echocardiography was used.

Statistical Analysis

All biochemical and functional assays were performed in mice obtained at 10 to 12 weeks of age along with mixed-gender controls. One-way ANOVA was used to test for overall significance, followed by the Student–Newman–Keuls test for multiple comparison testing between the various groups (Sigma Plot 11). Data are presented as means±SEM.
Results

Phosphorylation of Ser-282 Is Critical for Ser-302 Phosphorylation

We focused on three sites in the cardiac-specific insertion region of cMyBP-C to determine their roles in modulating cardiac function. Initially, to determine the substrate specificity of these sites by PKA, PKC, and CaMKII, recombinant human His-tagged C1-mol/L-C2 fragments of cMyBP-C were generated in which the three phosphorylatable sites were individually mutated to alanine, as well as a wild-type control (Figure 1A). Peptides were treated with PKA, PKC, or CaMKII, and their phosphorylation status was determined by Western blot analysis using site-specific cMyBP-C phospho-antibodies (Figure 1B, 1C). Experiments with PKA-mediated and PKC-mediated phosphorylation confirmed that PKA phosphorylates all three sites, whereas PKC phosphorylates only Ser-273 and Ser-302 (Figure 1C). At low Ca2+ concentrations, CaMKII targets Ser-302, whereas at high Ca2+ concentrations Ser-282 also can be a substrate (supplemental Figure I available online at http://www.circresaha.org), confirming that Ca2+-dependent CaMKII differentially phosphorylates Ser-282 and Ser-302. Phosphorylation at Ser-282 appears to be required for Ser-302 phosphorylation in vitro (Figure 1B, panel 4, antip302).

To explore further the site-specificity of Ser-273, Ser-282, and Ser-302 for PKA, PKC, and CaMKII, we infected neonatal rat cardiomyocytes with adenovirus expressing constitutively active (CA) and dominant negative (DN) CaMKII and PKCε adenoviruses (Ad) at MOI of 100 (n=5). Negative control using an empty vector for infection, isoproterenol-treated (ISO); 50 nmol/L ISO neonatal rat cardiomyocytes. H and I, At different time points after transverse aortic constriction (TAC) in mice, Western blot analysis of total heart proteins using site-specific phospho cMyBP-C antibodies performed Western blot analysis using our site-specific cMyBP-C phospho-antibodies and compared those results with isoproterenol-treated cardiomyocytes as a positive control for the PKA activation. We then performed Western blot analysis using our site-specific cMyBP-C phospho-antibodies and compared those results with isoproterenol-treated cardiomyocytes as a positive control.

Results show that overexpression of constitutively active CaMKIIε specifically increased Ser-302 phosphorylation compared to controls, confirming that Ser-302 is a substrate for CaMKIIε ex vivo, whereas the PKCε transfections confirmed that Ser-273 and Ser-302 are both PKC substrates (Figure 1D–1G). To demonstrate active regulation of these sites during cardiac stress, a transverse aortic constriction-induced pressure-overload mouse model was used. Western blot analyses, using site-specific phospho cMyBP-C antibodies, of neonatal rat cardiomyocytes infected with constitutively active (CA) and dominant negative (DN) CaMKIIε and (F and G) PKCε adenoviruses (Ad) at MOI of 100 (n=5). C. Negative control using an empty vector for infection, isoproterenol-treated (ISO); 50 nmol/L ISO neonatal rat cardiomyocytes. H and I, At different time points after transverse aortic constriction (TAC) in mice, Western blot analysis of total heart proteins using site-specific phospho cMyBP-C antibodies demonstrated that phosphorylation increased significantly after 30 minutes, but that Ser-282 phosphorylation was significantly reduced after 24 hours compared to Ser-273 and Ser-302 sites (n=6). *P<0.01 vs control or sham (n=5).

Figure 1. Differential substrate specificities and regulation of Ser-273, Ser-282, and Ser-302 phosphorylation. A, His-tagged C1-M-C2 recombinant human cardiac myosin-binding protein-C (cMyBP-C) peptide that includes the C1-M-C2 domains with the three phosphorylation sites. In the normal endogenous protein, each of these three serines is phosphorylatable, B, The ability of each residue to be phosphorylated by protein kinase (PK) A, Ca2+-calmodulin-activated kinase II (CaMKIIε), or PKCε is shown in (B) and (C), respectively. A dash indicates that the endogenous fragment with all three serines intact is present, and immunoblotting is indicated by “IB” (Western blotting). The mutation of serine (S) to alanine (A) at each residue is indicated as Ser to Ala. Phosphorylation of each residue with each enzyme was assayed using our phospho-residue–specific antibodies via Western blot analyses. Ponceau-S staining of the membranes confirmed equal amounts of protein were loaded. D and E, Western blot analyses, using site-specific phospho cMyBP-C antibodies, of neonatal rat cardiomyocytes infected with constitutively active (CA) and dominant negative (DN) CaMKIIε and PKCε adenoviruses (Ad) at MOI of 100 (n=5). C. Negative control using an empty vector for infection, isoproterenol-treated (ISO); 50 nmol/L ISO neonatal rat cardiomyocytes. H and I, At different time points after transverse aortic constriction (TAC) in mice, Western blot analysis of total heart proteins using site-specific phospho cMyBP-C antibodies demonstrated that phosphorylation increased significantly after 30 minutes, but that Ser-282 phosphorylation was significantly reduced after 24 hours compared to Ser-273 and Ser-302 sites (n=6). *P<0.01 vs control or sham (n=5).
the sites were differentially regulated in terms of their steady-state phosphorylation levels. These in vivo experiments are consistent with the hypothesis that Ser-282 phosphorylation is an early event in the stressed heart but does not resolve the question of whether its posttranslational modification is necessary for the subsequent phosphorylation of the other sites. To resolve this question, we turned to the intact animal and replaced endogenous cMyBP-C with protein that could not be phosphorylated at Ser-282.

Three Transgenic Mouse Models

Three separate constructs were used to generate multiple lines of TG mice to test the hypothesis that Ser-282 phosphorylation influenced the phosphorylation levels of residue 302 (Figure 2A, 2B). In the cMyBP-CWT mouse model, Ser-282 of cMyBP-C was mutated to a nonphosphorylatable alanine, preventing Ser-282 phosphorylation. In the cMyBP-CADA mouse model, the PKC sites (Ser-273 and Ser-302) were mutated to nonphosphorylatable alanines and Ser-282 was replaced with aspartate, resulting in a Ser-282 phospho-mimetic. In contrast, the third mouse model, cMyBP-CASD, contains charged residues (aspartates) at amino acids 273 and 302, mimicking constitutive PKC-mediated phosphorylation in the absence of phosphorylation at Ser-282, resulting in Ser-282 phospho-ablation. All TG mice were characterized before crossing into the cMyBP-C(t/t) background to assess any effects of TG expression. In each TG mouse model, we obtained three lines with variable transgene expression, resulting in 25% to 45% replacement of the endogenous cMyBP-C with TG protein (supplemental Figure II available online at http://www.circresaha.org). None of the lines showed any obvious phenotype or increased morbidity and
mortality from cMyBP-C knockout mice, whereas WT or DAD mice remained healthy. To confirm the incorporation of cMyBP-C in the heart, we performed Western blotting (Figure 2D). Using antibodies against the myc tag as well as to the first 14 N-terminal residues of cMyBP-C, the incorporation of the mutant cMyBP-C into the sarcomere was confirmed as well as the ability of the protein to maintain the proper stoichiometry by Western blotting (Figure 2D).

To determine if elimination of Ser-282 phosphorylation affected Ser-302 phosphorylation, we treated myofilaments derived from cMyBP-C\(^{\text{SAS}}\) hearts with PKA, PKC, and CaMKII and determined the phosphorylation status of each residue by Western analyses using the residue-specific phospho-antibodies (Figure 2E). As expected, the Ser-282 site-specific phospho-antibody showed no reactivity under any conditions with cMyBP-CSAS(t/t) derived protein. Strikingly, the incorporation of the mutant cMyBP-C into the sarcomere was confirmed as well as the ability of the protein to maintain the proper stoichiometry by Western blotting (Figure 2D).

Table 1. In Vivo Cardiac Function Assayed by M-Mode Echocardiography

<table>
<thead>
<tr>
<th></th>
<th>NTG (n=7)</th>
<th>WT(t/t) (n=7)</th>
<th>SAS(t/t) (n=12)</th>
<th>ADA(t/t) (n=8)</th>
<th>DAD(t/t) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSd (mm)</td>
<td>0.73±0.12</td>
<td>0.74±0.10</td>
<td>1.02±0.13</td>
<td>1.14±0.15</td>
<td>1.21±0.10</td>
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<tr>
<td>LVPW (mm)</td>
<td>0.80±0.06</td>
<td>0.79±0.12</td>
<td>0.87±0.13</td>
<td>0.96±0.10</td>
<td>1.04±0.18</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.86±0.17</td>
<td>3.34±0.32</td>
<td>4.29±0.34</td>
<td>4.33±0.38</td>
<td>4.53±0.38</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.63±0.23</td>
<td>2.85±0.38</td>
<td>3.07±0.38</td>
<td>3.40±0.32</td>
<td>3.46±0.51</td>
</tr>
<tr>
<td>FS (%)</td>
<td>32±4</td>
<td>28±5</td>
<td>24±5</td>
<td>22±4</td>
<td>24±7</td>
</tr>
<tr>
<td>LV VOLD (mm)</td>
<td>64±7</td>
<td>68±12</td>
<td>83±15</td>
<td>85±18</td>
<td>95±18</td>
</tr>
<tr>
<td>LV VOLS (mm)</td>
<td>26±6</td>
<td>31±9</td>
<td>44±13</td>
<td>48±11*</td>
<td>51±17*</td>
</tr>
<tr>
<td>LV EF (%)</td>
<td>60±5</td>
<td>54±8</td>
<td>48±8</td>
<td>44±7*</td>
<td>47±11</td>
</tr>
<tr>
<td>LV Mass (g)</td>
<td>134±17</td>
<td>134±31</td>
<td>153±29</td>
<td>184±21</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>25.9±0.36</td>
<td>26.3±0.39</td>
<td>24.95±0.51</td>
<td>26.17±0.37</td>
<td>25.6±0.46</td>
</tr>
</tbody>
</table>

ADA indicates Ala273-Asp282-Ala302; DAD, Asp273-Ala282-Asp302; EF, ejection fraction; FS, fractional shortening; IVSd, interventricular septum; LV, left ventricular; LVIDd, left ventricular inner diameter in diastole; LVIDs, left ventricular inner diameter in systole; NTG, nontransgenic; PW, ●●●; SAS, Ser273-Ala282-Ser302; VOLD, volume in diastole; VOLS, volume in systole; WT, wild-type.

Measurement values in mm were averaged from at least three separate cardiac cycles. All values given as mean±standard deviation.

*P<0.05 vs NTG.
†P<0.01 vs NTG.
‡P<0.001 vs NTG.
§P<0.05 vs t/t.
¶P<0.01 vs t/t.
‖P<0.001 vs t/t.
ingly, the loss of Ser-282 phosphorylation impacted significantly on the ability of PKA, PKC, and CaMKII to phosphorylate Ser-302 (Figure 2E). Specifically, CaMKII-mediated phosphorylation was completely abolished in the SAS myofilament preparations (Figure 2E and Figure 4). To determine whether increased Ca\(^{2+}\) concentrations could overcome this block in the phospho-ablated cardiac sample, 25 mmol/L Ca\(^{2+}\) was used with CaMKII to treat the cMyBP-CSAS(t/t) myofilaments. Western blot analyses showed that increased Ca\(^{2+}\) concentrations can stimulate Ser-282 phosphorylation in the NTG and cMyBP-C WT(t/t) myofilaments, whereas Ser-302 phosphorylation in the absence of Ser-282 phosphorylation was reduced, with the site being hypophosphorylated relative to normal cMyBP-C (Figure 2F).

Sarcomere Architecture in the SAS, ADA, and DAD Mutant Hearts

We reported previously that expression of cMyBP-C WT(t/t) and cMyBP-C AllP(t/t) effectively rescued the cMyBP-C(t/t) phenotype, but that expression of the nonphosphorylatable cMyBP-C AllP(t/t) failed to rescue the null, suggesting the necessity of cMyBP-C phosphorylation for normal sarcomere and cardiac function. To explore this observation at the individual residue level, we first determined whether the phospho-specific mutants incorporated normally into the sarcomere. Immunohistochemical analyses were performed with both anti-myc and anti-cMyBP-C antibodies (Figure 3A, 3B). Results show that SAS, ADA, and DAD proteins were incorporated normally into the sarcomere in the cMyBP-C WT(t/t) mouse background with striations indistinguishable from those seen in the NTG and cMyBP-C WT(t/t) sarcomeres (Figure 3A, 3B). Histological analyses showed that, as expected, the cMyBP-C WT(t/t) hearts displayed cardiac hypertrophy with fibrosis and disarray. However, there were no obvious abnormalities, fibrosis, calcification, or disarray observed in the cMyBP-CSAS(t/t) and cMyBP-CA(DA)(t/t) samples compared to NTG and cMyBP-C WT(t/t) controls (Figure 3C and 3D). In contrast with these data, the cMyBP-C DAD(t/t) mouse hearts showed fibrosis and disarray, mimicking the cMyBP-C WT(t/t) phenotype. Considering that this mouse contains the cMyBP-C phospho-mimetic in which residues 273 and 302 are constitutively charged, the data emphasize the importance of Ser-282 phosphorylation for cMyBP-C function. Transmission electron microscopy confirmed the previous observations of subtle organizational changes at the center of the sarcomere, at the M-line, and in the cMyBP-C-null mice, with the characteristic electron-dense M-line largely absent or irregular (Figure 3E). Consistent with the lack of disarray and fibrosis observed in the cMyBP-C WT(t/t), cMyBP-CSAS(t/t), and cMyBP-CA(DA)(t/t) hearts, the center of those sarcomeres contained a well-defined M line that was indistinguishable from those observed in normal sarcomeres in 81% to 91% of the sarcomeres. In contrast, 83% of the cMyBP-C DAD(t/t) sarcomeres displayed significant M-line abnormalities, suggesting that Ser-282 phosphorylation is necessary for the structural functions of cMyBP-C.
The heart-to-body weight ratios were normal in cMyBP-CSAS(t/t) (0.57 ± 0.02) and cMyBP-CADA(t/t) (0.57 ± 0.02) mice compared to cMyBP-C WT:(t/t) (0.56 ± 0.02) and NTG (0.50 ± 0.013) controls at 3 months. Consistent with the histology, cMyBP-CDAD(t/t) mouse hearts showed significantly increased heart-to-body weight ratios (0.70 ± 0.015, P < 0.0001 vs NTG), as did the cMyBP-C(t/t) hearts (0.81 ± 0.02, P < 0.0001 vs NTG),7 but with normal systolic function (Table 1). In contrast, cMyBP-CADA(t/t) mice showed normal heart-to-body weight ratios but reduced systolic function, suggesting that phospho-specific mutations have differential effects on cardiac structure and function. M-mode echocardiographic measurements showed that expression of normal cMyBP-C (cMyBP-CWT(t/t)) rescued the cMyBP-C(t/t) phenotype as previously shown (Table 1).7,9 In contrast to those data, cMyBP-CSAS(t/t), cMyBP-CDAD(t/t) and cMyBP-CADA(t/t) hearts showed only partial rescue of the null phenotype. Each model had significantly increased intraventricular septal thickness and left ventricular end-diastolic and end-systolic dimensions (Table 1), indicating that none was as effective at restoring normal cardiac hemodynamics as the WT protein (Table 1). The cMyBP-C-SAS(t/t), cMyBP-C-ADA(t/t), and cMyBP-C-CDAD(t/t) mice showed only partial rescue of the null phenotype. Each model had significantly increased intraventricular septal thickness and left ventricular end-diastolic and end-systolic dimensions (Table 1), indicating that none was as effective at restoring normal cardiac hemodynamics as the WT protein (Table 1). The cMyBP-C-SAS(t/t), cMyBP-C-ADA(t/t), and cMyBP-C-CDAD(t/t) hearts showed reduced fractional shortening, although only the cMyBP-C-ADA(t/t) hearts reached statistical significance. These data suggest that phosphorylation at Ser-273, Ser-282, and Ser-302, individually or in combination, is required for normal cardiac function to be maintained long-term.

β-AR Stimulation and cMyBP-C Phosphorylation

Phospho-ablation at Ser-282 attenuates Ser-302 phosphorylation, and we hypothesized that this would critically impact the ability of the heart to respond to β-AR stimulation. The cMyBP-C-SAS(t/t) hearts contain only cMyBP-CSAS cMyBP-C, allowing us to determine the functional consequence of the inability of Ser-282 to be phosphorylated in vivo during β-AR stimulation. We noted that Ser-273 appeared to be hyperphosphorylated in the cMyBP-C-SAS hearts at baseline, in contrast to the hypophosphorylation observed at Ser-302 (Figure 4A, 4B). At baseline, all groups showed normal systolic function (dP/dt max), suggesting that mutating the three selected phosphorylation sites does not affect systolic function. On β-AR agonist stimulation (dobutamine infusion), a reduced response was observed in all of the TG hearts, with the exception of those animals expressing the normal cMyBP-C protein (cMyBP-CWT(t/t)). The cMyBP-C-SAS(t/t) hearts further showed significantly decreased phosphorylation at Ser-282 during β-agonist stimulation compared to NTG and cMyBP-C WT(t/t) controls, confirming the importance of Ser-282 phosphorylation in mediating Ser-302 phosphorylation as well (Figure 4C). Interestingly, the mice containing phosphor-mimetics (cMyBP-C-ADA(t/t)) and phospho-ablation (cMyBP-C-ADA(t/t)) at residues 273 and 302 could not respond normally to dobutamine, emphasizing the importance of normal and, potentially, reversible phosphorylation at each of the three sites in mediating normal function and β-agonist=−induced stimulation.
Table 2. In Vivo Cardiac Hemodynamic Measurements

<table>
<thead>
<tr>
<th></th>
<th>NTG (n=5)</th>
<th>(t/t) (n=8)</th>
<th>WT((t/t) (n=6)</th>
<th>SAS((t/t) (n=6)</th>
<th>ADA((t/t) (n=5)</th>
<th>DAD((t/t) (n=5)</th>
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</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>Basal</td>
<td>Dobutamine (32 ng/g/min)</td>
<td>411 ± 8</td>
<td>385 ± 7*</td>
<td>400 ± 5</td>
<td>363 ± 7†</td>
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<tr>
<td></td>
<td>MAP (mm Hg/s)</td>
<td>Dobutamine (32 ng/g/min)</td>
<td>589 ± 10</td>
<td>533 ± 16*</td>
<td>587 ± 10‡</td>
<td>555 ± 20</td>
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<tr>
<td></td>
<td>Systolic pressure (mm Hg/s)</td>
<td>Dobutamine (32 ng/g/min)</td>
<td>72.8 ± 3</td>
<td>66.2</td>
<td>74.4</td>
<td>68.4</td>
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<tr>
<td></td>
<td>LVP (mm Hg/s)</td>
<td>Dobutamine (32 ng/g/min)</td>
<td>88.6 ± 7</td>
<td>76.5</td>
<td>88 ± 5</td>
<td>78.5</td>
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<td>LVP (mm Hg/s)</td>
<td>Dobutamine (32 ng/g/min)</td>
<td>85.8</td>
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<td>70.6</td>
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<td>dP/dtmin (mm Hg/s) Basal</td>
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<td>91 ± 4</td>
<td>105 ± 4‡</td>
<td>93 ± 3</td>
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<td>dP/dtmax (mm Hg/s) Basal</td>
<td>8611 ± 703</td>
<td>7853 ± 385</td>
<td>7842 ± 464</td>
<td>7472 ± 582</td>
<td>7058 ± 805</td>
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<td></td>
<td>dP/dtmax (mm Hg/s) Basal</td>
<td>19 480 ± 1227</td>
<td>14 858 ± 1362*</td>
<td>19 016 ± 664‡</td>
<td>15 283 ± 1426*</td>
<td>14 242 ± 1629*</td>
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<td></td>
<td>dP/dtmax (mm Hg/s) Basal</td>
<td>−10 008 ± 914</td>
<td>−4486 ± 224‡</td>
<td>−7720 ± 361¶</td>
<td>−5868 ± 483‡‡</td>
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<td>dP/dtmax (mm Hg/s) Basal</td>
<td>−14 548 ± 2091</td>
<td>−5225 ± 409*</td>
<td>−10 138 ± 952§</td>
<td>−8077 ± 495§§</td>
<td>−7143 ± 727‡‡</td>
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<td></td>
<td>LVEDP (mm Hg/s) Basal</td>
<td>7.2 ± 0.3</td>
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<td>5.1‡</td>
<td>10 ± 1</td>
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<td>LVEDP (mm Hg/s) Basal</td>
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<td>3.07</td>
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<td>dP/dt40 (mm Hg/s) Basal</td>
<td>8053 ± 508</td>
<td>7358 ± 350</td>
<td>7106 ± 294</td>
<td>7082 ± 618</td>
<td>6270 ± 1005</td>
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<td></td>
<td>Dobutamine (32 ng/g/min)</td>
<td>16367 ± 499</td>
<td>13 678 ± 995*</td>
<td>16 037 ± 284‡</td>
<td>13 536 ± 1140*</td>
<td>12 831 ± 1364*</td>
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</table>

ADA indicates Ala273-Asp282-Asp302; DAD, Asp273-Ala282-Asp302; dP/dtmax, maximum rate of heart contraction and relaxation; dP/dtmin, minimum rate of heart contraction and relaxation; EDP, end-diastolic pressure; LV, left ventricular; LVP, left ventricular pressure; NTG, nontransgenic; MAP, mean arterial pressure; SAS, Ser273-Ala282-Ser302; WT, wild-type.

Ventricular contraction and relaxation rates were measured at baseline and during β-agonist stimulation in the intact closed chest model, including telemetric measurements of heart rate, MAP, LV systolic pressure and LVEDP, LVP, +dP/dtmax, −dP/dtmin, and the rate of LVP increase determined at an LVP of 40 mm Hg (dP/dt40). Cardiovascular stress response to increasing doses of dobutamine was determined during 3-minute constant infusions (0.1 μL/min/g body weight), and data were analyzed using a PowerLab system (AD Instruments). Data are presented as mean ± SE.

*P < 0.05.
†P < 0.005 compared with NTG.
‡P < 0.05.
§P < 0.005.
¶P < 0.0005 compared with (t/t).

Discussion

Increasing data suggest that cMyBP-C phosphorylation can regulate myocardial function, sarcomere integrity, and, in some cases, can be cardioprotective. It is currently the only thick filament protein that is differentially phosphorylated by five important kinases (Figure 5) that can impact on cardiac contraction: PKA, PKC, CaMKII, PKD, and ribosomal s6 kinase. Our present study focused on deciphering the phosphorylation pattern of cMyBP-C and determining which phosphorylatable sites are necessary or perhaps sufficient for normal cardiac function. Although cMyBP-C is extensively phosphorylated under basal conditions, the level of cMyBP-C phosphorylation decreases in animal models during development of heart failure, myocardial injury, pathological hypertrophy, and myocardial stunning. Previously, to better-understand the mechanisms and significance of cMyBP-C phosphorylation, we established two TG mouse models to determine the necessity and sufficiency of cMyBP-C for normal cardiac function. Our findings showed that cMyBP-C phosphorylation is essential for normal cardiac function and that a phospho-mimetic at the Ser-273, Ser-282, and Ser-302 phosphorylation sites conferred cardioprotection against myocardial ischemia reperfusion injury in either the α-myosin heavy chain or β-myosin heavy chain backgrounds.

Phosphorylation of Ser-282 was a focus of the present study because phosphorylation levels of this residue are decreased in patients with heart failure and atrial fibrillation. Strikingly, Ser-282 is also selectively phosphorylated by ribosomal s6 kinase, which regulates myofilament function. In vitro studies demonstrated that phosphorylation at Ser-282 is a prerequisite for phosphorylation of the Ser-273 and Ser-302 sites. The data obtained from those studies also showed that the ability to phosphorylate Ser-273 and Ser-302 is markedly reduced when Ser-282 is mutated to alanine, suggesting that hierarchical phosphorylation may be involved in cMyBP-C-mediated regulation of contraction in cardiac muscle. In the present study, we removed the ability of Ser-282 to be phosphorylated to investigate the importance of Ser-282 phosphorylation in maintaining normal cardiac function at the whole organ function. Our data demonstrate that Ser-282 regulates the subsequent phosphorylation of Ser-302, but not Ser-273. We hypothesize that Ser-282 is both a regulatory and functional site for phosphorylation, with the cMyBP-C(DAD(t/t)) mice showing significant pathology at the light and ultrastructural levels (Figure 3D, E).

cMyBP-C is a substrate for PKC, which can phosphorylate Ser-273 and Ser-302 in cardiomyocytes. PKCε can also phosphorylate cMyBP-C, and this series of enzymes, some more selective than others, makes cMyBP-C phosphorylation exquisitely responsive to the changing environment. cMyBP-C phosphorylation, which is partially mediated by PKC, appears to play a critical role in cardiac function.
However, the roles of these sites in cardiac physiology have not been characterized. PKC phosphorylation mediates a major mechanism by which the myofilament modulates changes in myocardial function. 35 Gatul et al 19 obtained in vitro evidence that phosphorylation of Ser-282 may potentiate the PKC phosphorylation sites (Ser-273 and Ser-302), and thus phosphorylation could function as a switch that might also be triggered to mediate accelerated cardiac function during β-AR activation. Evidence from other studies suggests that cMMyBP-C phosphorylation influences actomyosin Mg2+ ATPase activity, the kinetics of cross-bridge cycling, and the rate of relaxation. 36-38 Therefore, the second objective of the study was to assess the necessity and sufficiency of PKC-mediated cMMyBP-C phosphorylation for sarcomeric integrity and for normal cardiac function. cMMyBP-C ADA:(t/t) and cMMyBP-C DAD:(t/t) hearts showed decreased hemodynamics and contractility relative to cMMyBP-C WT:(t/t) controls during β-AR stimulation because of the constitutive phosphorylation or phospho-mimetic “phosphorylation” (charged residues) of the PKC phosphorylation sites in cMMyBP-C.

Importantly, cMMyBP-C ADA:(t/t) mice unambiguously showed the effect of Ser-282 phosphorylation in the absence of PKC site phosphorylation on whole organ anatomy and function and partially rescued the cMMyBP-C C(t/t) phenotype. Conversely, despite the charged residues at Ser-273 and Ser-302, the cMMyBP-C DAD:(t/t) hearts displayed extensive damage compared to cMMyBP-C ADA:(t/t) and cMMyBP-C SAS:(t/t) mice and damage to cellular components (Figure 3) and had hypertrophy over time, mimicking the t/t null phenotype. Why do the cMMyBP-C DAD:(t/t) hearts display more overt abnormal pathology than the cMMyBP-C SAS:(t/t) mice when residue 282 is mutated in both? Overexpression of PKC isoforms in the heart causes hypertrophy in adult mice. 39 We hypothesize that mimicking constitutive activation of these two PKC sites in cMMyBP-C by replacing the two serines with aspartates in the cMMyBP-C DAD:(t/t) cardiomyocytes is detrimental to the hearts. In contrast, serines 273 and 302 in the cMMyBP-C SAS:(t/t) will not behave as if they were chronically phosphorylated; in fact, serine 302 will be hypophosphorylated in these hearts and thus not exhibit the effects of chronic phosphorylation, as manifested both by a hypertrophic response and a decreased M-line definition.

Conclusion

In conclusion, our data confirm the critical importance of Ser-282 in maintaining normal cardiac (Figure 3C, 3D) and sarcomeric (Figure 3E) architecture regardless of the phosphorylation status of the PKC sites. These data provide strong evidence that cMMyBP-C phosphorylation directly affects the heart’s contractile properties and sarcomere organization.

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Disclosure

None.

References


Novelty and Significance

What Is Known?

- Cardiac myosin binding protein-C (cMyBP-C) can be phosphorylated at multiple sites, with phosphorylation levels decreasing during cardiac stress and the development of heart failure.
- Three of the serines in cMyBP-C (Ser-273, Ser-282, and Ser-302) are substrates for protein kinase A, protein kinase C, protein kinase D, 90-kDa ribosomal S6 kinase, and Ca\(^{2+}\)-calmodulin-activated kinase II.
- It is unclear whether phosphorylation of these sites is functionally equivalent.

What New Information Does This Article Contribute?

- During cardiac stress, Ser-282 phosphorylation is differentially regulated, compared to the other two sites, suggesting that Ser-282 phosphorylation has a unique role in myocardial function.
- A phosphorylated serine at position 282 is required for Ser-302 phosphorylation, suggesting that Ser-282 phosphorylation is a critical determinant of cMyBP-C phosphorylation at Ser-302.

- Ser-302 is differentially phosphorylated by calmodulin-dependent protein kinase II.
- The data suggest that Ser-282 phosphorylation may act in a regulatory manner. In addition, Ser-302 is also a functional site, partially mediating the contractile response to \(\beta\)-adrenergic stimulation.

We previously determined that cMyBP-C phosphorylation is vital for normal cardiac function and that phosphorylation of the Ser-273, Ser-282, and Ser-302 sites conferred cardioprotection against ischemic reperfusion injury in either the \(\alpha\)-myosin heavy chain or the \(\beta\)-myosin heavy chain backgrounds. In the present study, we discovered that Ser-282 phosphorylation is a critical determinant of Ser-302 phosphorylation and that Ser-282 partially regulates the contractile response to \(\beta\)-adrenergic stimulation in vivo. Future studies will define the functional and mechanistic effects of cMyBP-C phosphorylation at individual sites in mediating the interactions of cMyBP-C with other sarcomeric proteins, as well as posttranslational effects on sarcomeric integrity and myocardial function.
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Running title: Phosphorylation of Cardiac Myosin Binding Protein-C

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Online Supplement Figure I. CaMKII requires high levels of Ca\textsuperscript{2+} to phosphorylate Ser-282 in cMyBP-C. Recombinant His\textsubscript{6}-tagged C1-M-C2 peptide of human cMyBP-C (100 pmol) was incubated with pre-activated CaMKII at either 2mM or 25mM CaCl\textsubscript{2}, 1.2\mu M CaM, 200 \mu M ATP for 10 min at 30°C, with recombinant Calmodulin-dependent Protein Kinase II (CaMKII); New England Biolabs Cat. No. P6060S or the PKA catalytic subunit (Millipore) for 20 min at 30°C in a final volume of 50 \mu l (30mM Tris pH 7.4; 15mM MgCl\textsubscript{2}; 1mM DTT; 100\mu M ATP). Proteins were subsequently resolved on 12% acrylamide gels, transferred nitrocellulose membranes and immunoblotted with anti-pSer273 (1:3000), anti-pSer282 (1:3000) or anti-pSer302 (1:10000) phospho-specific antibodies or anti-cMyBP-C\textsuperscript{2-14} antibody. Results show that Ser-302 is preferentially phosphorylated by the enzyme at low Ca\textsuperscript{2+} concentrations while at higher Ca\textsuperscript{2+} concentrations Ser-282 is phosphorylatable by CaMKII.
Online Supplement Figure II. Transgenic (TG) expression of cMyBP-C mutant lines.

A. Southern blot analysis of genomic DNA from non-transgenic (NTG), wild-type (WT) TG line 21, three lines from cMyBP-C\textsuperscript{SAS} (lines 142, 153 and 155), three lines of cMyBP-C\textsuperscript{ADA} (lines 14, 22 and 25) and three lines of cMyBP-C\textsuperscript{DAD} (lines 212, 214 and 151). Ten µg of total genomic DNA was digested with EcoRI, separated on agarose, transferred to nitrocellulose and probed with an α-myosin heavy chain promoter specific probe (1000 bp), which recognize both endogenous and TG sequences. Quantitation of the signal indicated that the cMyBP-C\textsuperscript{SAS}, cMyBP-C\textsuperscript{ADA} and cMyBP-C\textsuperscript{DAD} lines used for the subsequent studies inherited 5, 4 and 2 transgene copies, respectively, compared to 6 copies in the WT TG line 21. Expression of the cMyBP-C mutant constructs did not result in any detectable embryonic lethality. B-C, Northern blot analyses of total mRNA from left ventricles derived from 3-month old NTG, WT-Tg line 21 and 3 lines from the cMyBP-C mutant mice. To confirm expression of the intact and correctly sized transcript, Northern blot analysis was performed with cMyBP-C and GAPDH probes. Results show that compared to the NTG, significantly increased cMyBP-C transcript levels were observed in the selected TG lines (*\(P<0.01\), n=3). D, Representative SDS-PAGE analysis shows the conservation of the sarcomeric protein levels in these mice compared with a NTG sample. Five µg of total myofilament proteins were loaded on a 4-15% gradient gel (Bio-Rad, Cat. No. 456-1083) and stained with SYPRO-RUBY (Bio-Rad, Cat. No. 170-3138). E, Western blot analysis shows the absence of TG cMyBP-C in the NTG hearts and the presence of myc-tagged Tg cMyBP-C in TG mice. cTnI was used as a loading control (Cell Signaling Technology, Cat. No. 4002S). F-H, Total cMyBP-C levels as well as the presence of myc-tagged cMyBP-C protein was confirmed by Western blot analysis. 5 µg of total myofilament proteins were loaded from the respective lines on the same SDS-PAGE for Western blot analysis using monoclonal anti-Myc tag antibody (Roche, Cat. No. 11667149001). NTG hearts do not express the Myc-tagged cMyBP-C.
The purpose of loading total myofilament proteins from both the single TG lines and the TG line in the (t/t) background mice on the same gel, side by side, was to normalize the loading and allow us to calculate the cMyBP-C Myc-tag replacement. In the homozygous (t/t) background, TG cMyBP-C levels are equal to 100% as there was no trace of cMyBP-C determined in the (t/t) background (Figure 2). TG Myc-tagged cMyBP-C was first normalized with α-actin (TG Myc/actin). TG Myc-tagged cMyBP-C in the (t/t) background was then normalized with α- actin (TG Myc(t/t)/actin). Then to obtain the amount of replacement in the transgenic lines, the value of [TGMyc/actin]/[TG Myc(t/t)/actin]*100 was determined. The data obtained from these analyses were used to determine the level of cMyBP-C mutant replacement in the TG lines, compared to the expression in the cMyBP-C null (t/t) background. α- actin was used as a loading control (Sigma, Cat. No. A2172).

References: