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PKCε Increases Phosphorylation of the Cardiac Myosin Binding Protein C at Serine 302 both in Vitro and in Vivo†

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Abstract

Cardiac myosin binding protein C (cMyBPC) phosphorylation is essential for normal cardiac function. Although PKC was reported to phosphorylate cMyBPC in vitro, the relevant PKC isoforms and functions of PKC-mediated cMyBPC phosphorylation are unknown. We recently reported that a transgenic mouse model with cardiac-specific overexpression of PKCε (PKCε TG) displayed enhanced sarcomeric protein phosphorylation and dilated cardiomyopathy. In the present study, we have investigated cMyBPC phosphorylation in PKCε TG mice. Western blotting and two-dimensional gel electrophoresis demonstrated a significant increase in cMyBPC serine (Ser) phosphorylation in 12-month-old TG mice compared to wild type (WT). In vitro PKCε treatment of myofibrils increased the level of cMyBPC Ser phosphorylation in WT mice to that in TG mice, whereas treatment of TG myofibrils with PKCε showed only a minimal increase in cMyBPC Ser phosphorylation. Three peptide motifs of cMyBPC were identified as the potential PKCε consensus sites including a 100% matched motif at Ser302 and two nearly matched motifs at Ser811 and Ser1203. We treated synthetic peptides corresponding to the sequences of these three motifs with PKCε and determined phosphorylation by mass spectrometry and ELISA assay. PKCε induced phosphorylation at the Ser302 site but not at the Ser811 or Ser1203 sites. A S302A point mutation in the Ser302 peptide abolished the PKCε-dependent phosphorylation. Taken together, our data show that the Ser302 on mouse cMyBPC is a likely PKCε phosphorylation site both in vivo and in vitro and may contribute to the dilated cardiomyopathy associated with increased PKCε activity.

Ventricular cardiomyocytes express multiple protein kinase C isoforms, including PKCα, PKCε, PKCδ, and PKCλ (1–3), which have been suggested to play key roles in adaptive and maladaptive cardiac responses and to contribute to the cardiomyocyte hypertrophic response following prolonged pathologic stresses (4–6). Among them, PKCε has a unique property of translocating to the myofilaments upon activation (3, 7) and potentially phosphorylating certain sarcomeric proteins such as TnI (8). During pathologic hypertrophy and heart failure, PKCε activity is often elevated (9–11) and has been linked to sarcomeric protein phosphorylation, β-adrenergic stimulation induced apoptosis, and repression of AKT phosphorylation mediated survival pathways (12–14). Our group previously characterized a transgenic mouse model overexpressing constitutively active PKCε in the heart at physiologically relevant levels (15, 16). These TG[1] mice developed a dilated cardiomyopathy, which became evident at 9–12 months of age. Along with the development

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[1]Abbreviations: cMyBPC, cardiac myosin binding protein C; TG, transgenic; WT, wild type; MS, mass spectrometry.

of hemodynamic and mechanical abnormalities, there was an increase in phosphorylation of two important sarcomeric proteins, cTnI and cTnT. In this study, we further evaluated the effect of PKCε in these mice on another important sarcomeric protein that is also potentially subjected to phosphorylation in vivo, cMyBPC.

Many pathologic features of heart failure are linked to the structural and functional alterations of the sarcomere (17, 18). In addition to contractile proteins, the sarcomere contains accessory proteins that are involved in assembly, maintenance of structural integrity, and regulation of contractile activity. Mutation of the genes encoding these proteins may cause either a hypertrophic or dilated cardiomyopathy. One of these proteins is cMyBPC, a polypeptide with a molecular mass (MW) around 135 ± 15 kDa. It is located in the C zone of the sarcomere and is known to bind myosin by interacting with the light meromyosin (19, 20) and the S2 subdomain (21, 22) and to bind titin at its C terminus (23, 24). Previous studies have demonstrated the essential role of cMyBPC in maintaining proper thick filament structure and cardiac contractility both in vitro and in vivo (19, 25, 26). cMyBPC-null mice exhibit remarkable myocardial hypertrophy with myofilament disarray and severely impaired cardiac contractility (27).

In several animal models of pathologic hypertrophy and heart failure, the total phosphorylation of the cMyBPC was decreased (28), suggesting a role of cMyBPC phosphorylation in the modulation of cardiac function under chronic pathologic stimuli. The mechanism underlying this observation and the physiological significance are unclear at this time. Early in vitro studies on purified myofibrillar proteins have demonstrated that cMyBPC is a potential substrate for both PKA (29–33) and PKC (34). Three potential PKA phosphorylation sites have been identified at the N terminus of chicken cMyBPC (Ser 265, Ser 300, and Tyr 274) by in vitro phosphorylation (35). These sites are all within the conserved cMyBPC motif between the C1 and C2 domains, and it has been proposed that phosphorylation of cMyBPC leads to release of S2, which makes myosin cross-bridges take on a position more favorable for actin binding (36). The importance of PKA-mediated phosphorylation on cMyBPC has been further established by the study of TG mice with nonphosphorylatable mutations in all potential PKA phosphorylation sites ($cMyBPC^{ALLP-}$) which showed contractile dysfunction with myofibrillar disarray (28). Although cMyBPC is also a good substrate for PKC, there are limited data on PKC-mediated phosphorylation of cMyBPC. In vitro phosphorylation studies of purified cMyBPC suggest that PKC could potentially phosphorylate the above two Ser PKA phosphorylation sites in vitro (35). However, the related isoforms and the functional implication of PKC-mediated phosphorylation on cMyBPC remain unclear at this time. In this study, we demonstrated that Ser phosphorylation of cMyBPC was significantly increased in the PKCε TG mice in vivo, and we have defined Ser302 as a target phosphorylation site for PKCε.

EXPERIMENTAL PROCEDURES

Sample Preparation

Cardiac myofibrillar protein purification from the ventricle tissue is as described previously (15). Briefly, frozen myocardium was homogenized in isolation buffer (in mmol/L: 100 KCl, 10 imidazole, 1 $MgCl_2$, 2 EGTA, 4 Na_2ATP, pH 7.2) plus 1% Triton X-100 on ice and subjected to low-speed centrifugation at 800 rpm at 4 °C. The pellet was washed twice with the isolation buffer and then three times with standard relaxing buffer [SRB, in mmol/L: 20 Tris-HCl (pH 7.4), 2 EDTA, 10 EGTA, 320 sucrose, 0.3 PMSF, 20 μg/mL leupeptin] and stored in the SRB for Western blots and PKCε kinase assay studies. Protein extraction for two-dimensional (2D) gel electrophoresis was done according to previously described methods (37–39). Briefly, myocardial tissue (~10–30 mg) from mouse ventricle was powdered in lysis buffer (6 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris, pH 8.5)

with a weight:volume ratio of 1:20. The mixture was sonicated and then incubated for 1 h at 4 °C. This was repeated twice, and then the mixture was centrifuged at 16200*g* for 30 min. The supernatant was collected and stored at –80 °C for later use.

Western Blotting

For 1D gel analysis, sample proteins were mixed with 10 mM β-mercaptoethanol and denatured by incubating in boiling water for 5 min before separation with 7.5% SDS–PAGE at 30 mA. The proteins separated by 1D or 2D gel were transferred to nitrocellulose at 100 V for 1 h at room temperature. Primary antibodies, rabbit anti-phosphoserine PKC substrate (p-SerPKC) polyclonal antibody, mouse anti-phosphotyrosine (p-Tyr) monoclonal antibody, and rabbit anti-phosphothreonine (p-Thr) monoclonal antibody, were purchased from Cell Signaling Technology (Beverly, MA; 1:2000 dilution). The rabbit anti-cMyBPC (1:10000) known to detect the N terminus of cMyBPC (27, 40, 41) was kindly provided by Dr. Richard L. Moss. Donkey anti-rabbit/mouse IgG conjugated to horseradish peroxidase (HRP) (Pierce Biotechnology, Rockford, IL) was used as secondary antibody, and the HRP activity was visualized with SuperSignal chemiluminescent substrate (Pierce Biotechnology, Rockford, IL).

2D Gel Electrophoresis

2D gel analysis was performed as previously described (37–39) with modifications to optimize the separation of large proteins. Protein lysate (as described above) was adjusted with lysis buffer to a final concentration of 5 mg/mL determined by the Bio-Rad RC/DC kit (Bio-Rad, Hercules, CA). Twenty microliters of protein sample (100 μg) was labeled with 200 pmol of Cy3 fluorescent dye (GE Healthcare, Piscataway, NJ). The labeling was carried out in the dark for 2 h at 4 °C and stopped by the addition of 0.25 μL of 0.01 M L-lysine solution (42). The fluorescence-labeled proteins were first separated by p*I* using immobilized pH gradient (IPG) strips (24 cm, PI 3–11; GE Healthcare) and then separated by molecular mass by SDS–PAGE. Briefly, fluorescence-labeled proteins were adjusted with Destreak Rehydration buffer (GE Healthcare), and IEF was performed for 50000 total v•h at 20 °C in an IEF cell (Bio-Rad). The regions on the IPG strips containing cMyBPC proteins (75 mm, p*I* 5.5– 8.0) were excised and further focused for 10000 v•h in a 7 cm focusing tray at 6000 V. After reduction and alkylation, the IPG strips were loaded on 4–12% Novex Bis-Tris gels (Invitrogen, Carlsbad, CA) using the Xcell SureLock system (Invitrogen). The SDS–PAGE was run at 150 V. All electrophoresis procedures were performed in the dark. At the end of the electrophoresis, gel slabs were washed with deionized and distilled water (dd H_2O) and Cy3-labeled proteins visualized using a Typhoon 9410 scanner (GE Healthcare). Western blotting was then performed as described above.

Peptide Synthesis

Eighteen amino acid peptides were prepared in the protein research laboratory at UIC using FMOC chemistry on a solid-phase peptide synthesizer (Symphony; Protein Technologies, Inc., Tucson, AZ) as described previously (43). The peptide was characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry [Voyager, DE PRO; Applied Biosystems (44)].

PKCε Assay

The method is a modification of the back-phosphorylation method described previously (15). Sample preparations from frozen ventricular tissue (~20 μg), synthesized peptides (70 μg), or both were mixed with a 20 μL reaction cocktail containing 1 pmol of ATP (Sigma), 0.64 μg of PKCε, PKCα, or PKCδ (human recombinant, Invitrogen), 16.75 μL of SRB, and 1.75 μL of micelle mix [a mixture containing 23 μL of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-

(phospho-L-serine), 12 μL of 1,2-dioleoyl-*sn*-glycerol, and 0.5 μL of Triton X-100]. For PKCα assay, $CaCl_2$ was also added to the reaction cocktail to a final concentration of 200 μM. The mixture was incubated for 20 min at 30 °C, followed by ELISA, MS, or SDS–PAGE.

Mass Spectrometry (MS) To Detect Phosphorylation on the Peptide

MS was performed using MALDI-TOF mass spectrometry to detect peptide phosphorylation according to their mass change as described (44).

ELISA for Detection of Phosphorylated S302 Peptide

ELISA was performed according to standard protocols (45, 46). Briefly, 2.5 μM synthetic peptides prepared in carbonate buffer (pH 9.6) or control samples were incubated on microtiter plates overnight at 4 °C. Wells were then washed three times with PBS containing 0.5% Tween-20 and blocked with BSA (10 mg/mL in PBS-Tween at 37 °C) for 1 h. After washing, p-SerPKC antibody, diluted in PBS-Tween at 1:1000, was incubated in each well for 2 h at 37 °C. After being washed three times with PBS-Tween, the wells were incubated with anti-rabbit IgG conjugated with alkaline phosphate (Sigma) at 1:500 dilution in PBS-Tween for 2 h at 37 °C. Finally, 50 μL of enzyme substrate (fast *p*-nitrophenyl phosphate tablet sets; Sigma) prepared in water was added for 10 min at 37 °C. The plate was read at 405 nm using a micotiter plate reader (Statfax-2100; Awarnes Technologies, Inc.).

RESULTS

Endogenous Ser Phosphorylation of cMyBPC, but Not Tyr or Thr Phosphorylation, Was Increased in the PKCε TG Mouse

The PKCε mice develop overt dilated cardiomyopathy at 9–12 months of age. In order to investigate phosphorylation of the cMyBPC at this stage, Western blotting using the p-SerPKC antibody was performed on ventricular myofibrillar proteins purified from 12-month-old WT or TG hearts. A phosphorylated protein was identified at 150 kDa whose MW was consistent with that of the mouse cMyBPC which was also identified by Western blotting using the anti-cMyBPC antibody. The Ser phosphorylation of this protein was significantly increased (to about 2.1-fold) in the PKCε TG heart compared with that in the WT heart (Figure 1A–C).

We determined the Ser phosphorylation levels of cMyBPC in the PKCε TG and WT mice using 2D gel electrophoresis on the whole cell protein lysate to further determine the identity of the 150 kDa protein identified on the 1D gel. Equal amounts of Cy3-labeled cell lysate from the WT or TG hearts were run on separate gels, followed by simultaneous protein transfer to the same PVDF membrane and Western blotting using the p-SerPKC antibody. The phosphorylated protein detected at 150 kDa by the p-SerPKC antibody was distributed across a wide pH range but at the acidic end of the spectrum as expected, and its distribution was spatially identical to that of the cMyBPC identified later by the generic cMyBPC antibody after membrane stripping. This finding further confirms that the phosphoprotein identified at 150 kDa is indeed cMyBPC. In addition, the Ser phosphorylation of the cMyBPC in the TG mice was also increased compared to that in the WT mice (Figure 1D), which was consistent with the finding from the 1D study described above.

Western blotting on the purified myofibrillar proteins with the p-Tyr antibody failed to detect any phosphorylated proteins at 150 kDa in either WT or TG mice (Figure 2A). Although Western blotting with the p-Thr antibody detected a phosphorylated protein at ~150 kDa in myofibrils from both WT and TG hearts (Figure 2A), there was no statistical

difference (n = 3; paired, one-tailed t-test, p = 0.3213) in phosphorylation between the preparations (Figure 2B). These results suggest that while the Thr residues of cMyBPC might be endogenously phosphorylated in the mouse cMyBPC, the phosphorylation level is likely unchanged in the WT or TG mice. These findings are consistent with the consensus that PKC, when activated, mainly phosphorylates substrates containing Ser or Thr residues. The Tyr or Thr residues of the cMyBPC are unlikely target phosphorylation sites for PKCε, implicating the specificity of PKCε phosphorylation on the Ser residues of cMyBPC.

PKCε Phosphorylated cMyBPC in Vitro and the Endogenous Ser Phosphorylation of the cMyBPC Was Increased to Near Saturation in the PKCε TG Mice

In order to determine the endogenous phosphorylation level of the Ser residues that are potential PKCε phosphorylation sites, purified cardiac myofibrillar proteins from WT or TG hearts were treated with PKCε in vitro, followed by Western blotting using the p-SerPKC antibody. The membrane was then stripped and blotted for cMyBPC. Serine phosphorylation of the cMyBPC in the WT hearts was significantly increased after PKCε treatment by 2.8-fold (Figure 3). In contrast, there was only about 30% increase in Ser phosphorylation in the TG hearts after the PKCε treatment (Figure 3). These findings indicate that PKCε phosphorylates Ser residue(s) of cMyBPC in vitro, and the endogenous phosphorylation level of this (these) potential PKCε phosphorylation site(s) is significantly increased in the PKCε TG mice to near saturation as analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test ($p < 0.05$).

The Serine Residue at Position 302 (Ser302) Was Identified as the Potential Site for Increased Ser Phosphorylation of cMyBPC in the PKCε TG Mice

In order to identify the specific Ser residue(s) that contribute(s) to the increased Ser phosphorylation of cMyBPC in the PKCε TG mice, we first performed NetPhos analysis (NetPhos 2.0 server, CBS, DTU http://www.cbs.dtu.dk), which produces neutral network predictions for Ser, Thr, Tyr phosphorylation sites in eukaryotic proteins (47), and identified over 40 Ser sites on the cMyBPC that could potentially be phosphorylated in vivo. However, among these, only one Ser site, located at position 302 on mouse cMyBPC is within a 100% matched consensus motif for the p-SerPKC antibody. Two other Ser residues located at position 811 and 1203 sites that have very similar motifs were also identified (Figure 4A).

Three 18 amino acid peptides containing the above Ser residues were synthesized using FMOC chemistry on a solid-phase peptide synthesizer (Symphony; Protein Technologies, Inc.) according to the corresponding amino acid sequence of cMyBPC (48). A mutant peptide with substitution of an Ala for the Ser302 (S302A) was also generated. The peptides were then treated with PKCε, followed by MALDI-TOF mass spectrometry, to detect phosphorylation according to their mass change, as phosphorylation of one residue on the peptide adds about 80 Da to the peptide mass. As shown in Figure 4B, the S302 peptide contains two Ser sites. After PKCε treatment, a mass shift indicated the addition of a single phosphate group (Figure 4B). The phosphorylation of this site was abolished after mutation of the Ser302 to Ala, indicating that Ser302 is the site phosphorylated by PKCε in vitro (Figure 4B). No mass shift was seen following PKCε treatment of the peptides containing Ser811 or Ser1203, suggesting that they are unlikely phosphorylation sites for PKCε (Figure 4C).

The PKCε -treated samples of S302 peptide, S302A peptide, and controls were then coated on the wells of a microtiter plate and enzyme-linked immunosorbent assay (ELISA) was performed using the p-SerPKC antibody. Enzymatic substrate activity was significantly increased in the PKCε -treated S302 peptides but not in the PKCε -treated S302A peptide or controls (Figure 5), indicating successful detection of binding of p-SerPKC antibody to the

phosphorylated S302 peptide which was abolished after the point mutation of Ser302 to an Ala.

The in vitro PKCε kinase competition assays (Figure 6) showed that the synthetic peptide S302 significantly (as analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test, $n = 3$, $p < 0.05$) competes off the PKCε -induced serine phosphorylation of the intact mouse cMyBPC. In contrast, neither a structurally unrelated serine-containing control peptide TKR (TKRKDEECSTTEHPYKKPYM) nor the structurally unrelated S302A mutant peptide showed this competition effect on cMyBPC serine phosphorylation. This result strongly suggests that the intact mouse cMyBPC shares the same in vivo phosphorylation motif for PKCε as that of the peptide S302.

Interestingly, we found that, like PKCε, another novel PKC subfamily isoform PKCδ also could significantly phosphorylate cMyBPC S302 in vitro (Figure 7), whereas the conventional PKC subfamily isoform PKCα was not able to phosphorylate cMyBPC S302 in vitro (Figure 7). This result implicated the potential roles of novel PKC subfamily members in the Ser phosphorylation of cMyBPC in vivo.

DISCUSSION

Cardiac MyBPC is an important sarcomeric protein that regulates thick filament integrity and contractility. Earlier studies have demonstrated that it can be phosphorylated by both PKA and PKC in vitro (29, 34, 35), suggesting a potential role of phosphorylation in regulating its function. However, its in vivo phosphorylation status, the functional impact of its phosphorylation, and the underlying regulatory mechanism are still poorly understood. While we are beginning to understand the impact of PKA-mediated phosphorylation of cMyBPC on cardiac function from the cMyBPC^{ALLP-} TG mice (28), the functional role of PKC-mediated phosphorylation is still largely unknown. In this study, we have provided evidence that cMyBPC was endogenously phosphorylated under normal physiologic conditions and for the first time demonstrated that its Ser phosphorylation, not Tyr or Thr phosphorylation, was increased in a TG mouse model of dilated cardiomyopathy and heart failure associated with elevated PKCε. Furthermore, we have suggested that Ser302 is a target phosphorylation site on the cMyBPC for PKCε in vivo and in vitro.

PKCε has the unique property of translocating to the myofilaments and phosphorylating sarcomeric proteins upon activation. This fact, coupled with the observation that PKCε is activated during pathologic hypertrophy and heart failure (9, 11, 49), provides the rationale for the generation of the PKCε TG mice used in this study which developed marked cardiac dilation and dysfunction at 9–12 months of age. Data presented in this study suggest that cardiac restricted PKCε overexpression increases site-specific cMyBPC phosphorylation in this model. This is based on the results from 1D and 2D gel analysis showing that Ser phosphorylation of the cMyBPC was increased in the PKCε TG mice. The demonstration that Ser302 of cMyBPC is a potential target phosphorylation site for PKCε is based on the findings that Ser302 is located within the only motif in mouse cMyBPC that is identical to the consensus motif recognizable by the p-Ser antibody, and PKCε was able to phosphorylate Ser302, but not other sites with similar motifs, when synthetic peptides containing these sites were treated with PKCε in vitro. In addition, the synthetic peptide S302, but not its mutant peptide S302A, could compete off the PKCε-induced phosphorylation of the mouse cMyBPC, which suggested that cMyBPC shares the same phosphorylation site for PKCε as that of peptide S302. Although these findings do not definitively establish that the increased phosphorylation of Ser302 in vivo was due to direct PKCε phosphorylation (as opposed to compensatory phosphorylation by other kinases (i.e., PKA), the above in vitro findings coupled with the identification of enhanced in vivo

phosphorylation suggest a potential role for PKCε-mediated cMyBPC phosphorylation in the progressive myopathy seen in the PKCε TG mice.

It is noteworthy that cMyBPC is not the only sarcomeric protein in the PKCε mice whose phosphorylation is altered. As previously reported (15), phosphorylation of the TnI and TnT was also increased. Subsequently, our group has reported that TG mice with a mutation of one of the PKC phosphorylation sites on cardiac TnI (S43,45A) exhibit improved cardiac performance in vivo (50) and are partially protected from the deleterious effects of PKCε overexpression (51), suggesting that PKC-dependent phosphorylation of TnI contributes to the decline in function seen in the PKCε mice. While it is possible that the partial response is a reflection of incomplete protein exchange, it is more likely that the myopathy is caused by the complex contribution of several other factors including parallel myofilament protein abnormalities. Our results support this hypothesis by demonstrating that the phosphorylation of cMyBPC is also increased in these PKCε mice. The role of cMyBPC phosphorylation cannot be dissociated from the other sarcomeric proteins already identified in these PKCε TG mice. However, identification of the potential PKCε-dependent phosphorylation site on cMyBPC makes targeted genetic manipulation of this sarcomeric protein possible, which may shed a light on the independent contribution of cMyBPC phosphorylation to cardiac muscle dysfunction.

Since there are multiple potential phosphorylation sites on the cMyBPC (37), one concern is whether other sites besides Ser302 exist on cMyBPC that contribute to the increased Ser phosphorylation in the PKCε mice. Findings from the previous studies and ours suggest that this is unlikely. First, the in vitro PKC and PKA phosphorylation studies on cMyBPC demonstrated that there are limited potential Ser phosphorylation sites on the chicken cMyBPC (35), which was also confirmed by the recent in vivo studies on multiple TG mouse lines using IEF gel analysis (28, 52). In mouse cMyBPC, besides Ser302 (corresponding to Ser300 in chicken cMyBPC), there are only two more potential PKA Ser phosphorylation sites reported, which are Ser residues located at positions 263 and 272 (corresponding to Ser265 and Tyr274 in chicken cMyBPC). These sites are within sequence motifs that share no similarity to the consensus motif for the p-SerPKC antibody. It is noteworthy that our 2D gels showed increased intensities of p-Ser in multiple cMyBPC spots from PKCε TG samples compared to the WT, which was consistent with a previous report documenting the presence of multiple forms of canine or rat cMyBPC with different p*I* values (37). Both studies might be interpreted to suggest the presence of additional posttranslational modifications on cMyBPC including phosphorylation by either PKCε or other kinases, which may modify the p*I* values of cMyBPC in addition to Ser302 phosphorylation in vivo. We have not yet identified the peptide sequence for those spots or the potential kinases responsible for the increased phosphorylations. On the other hand, even if other less prominent phosphorylation sites coexist on the cMyBPC in the PKCε TG mice in addition to the Ser302 site, this does not diminish the significance of Ser302 phosphorylation by PKCε.

Currently, it is well accepted that cMyBPC phosphorylation regulates cardiac contractile function. However, its exact role during the development and progression of physiological vs pathologic hypertrophy and heart failure and the underlying mechanisms are still largely unknown. The phosphorylation status of cMyBPC might be different in diverse models of pathological hypertrophy and heart failure, or even different in the same model at differential stages of the pathological process. Recently, it was reported that under prolonged pathologic stimuli such as aortic banding, cMyBPC phosphorylation identified by IEF gel analysis was decreased. However, early during the pathologic stress, cMyBPC phosphorylation was not significantly changed (52). Since the cMyBPC phosphorylation status is affected by both PKA and PKC pathways, this change in phosphorylation may

represent a net result of the differential influences of these kinases during the transition from adaptation to maladaptation. In addition, in some models of chronic pathologic hypertrophy, dilated cardiomyopathy, and heart failure (9, 11, 49), other PKC isoforms, such as PKCα and PKCδ, were also found to be upregulated. Whether or not the cMyBPC is also a substrate for these PKC isoforms is unknown. Thus, the overall physiologic relevance and phosphorylation status of cMyBPC are likely determined by the counterbalance of different kinases targeting different phosphorylation sites. It is certainly plausible that the progression of cardiac muscle dysfunction is characterized by a decrease in PKA and an increase in PKC-dependent phosphorylation. Isolating the functional significance of a single phosphorylation site on cMyBPC, especially in light of changes in the phosphorylation status of other sarcomeric proteins, is clearly a challenging undertaking and will probably require the generation of a set of targeted transgenic mouse models with cardiac-specific overexpression of cMyBPC 302 mutants that would both simulate and prevent site-specific phosphorylation.

In summary, we have demonstrated that, in a murine model of dilated cardiomyopathy induced by overexpression of PKCε, the Ser phosphorylation of cMyBPC was increased, and our results suggest that Ser302 on the cMyBPC is a target PKCε phosphorylation site. The functional impact of this finding in these TG mice cannot be dissociated from that credited to the posttranslational modifications of the other sarcomeric proteins already identified, but the data suggest that cMyBPC phosphorylation may contribute to the dilated cardiomyopathy associated with elevated PKCε levels.

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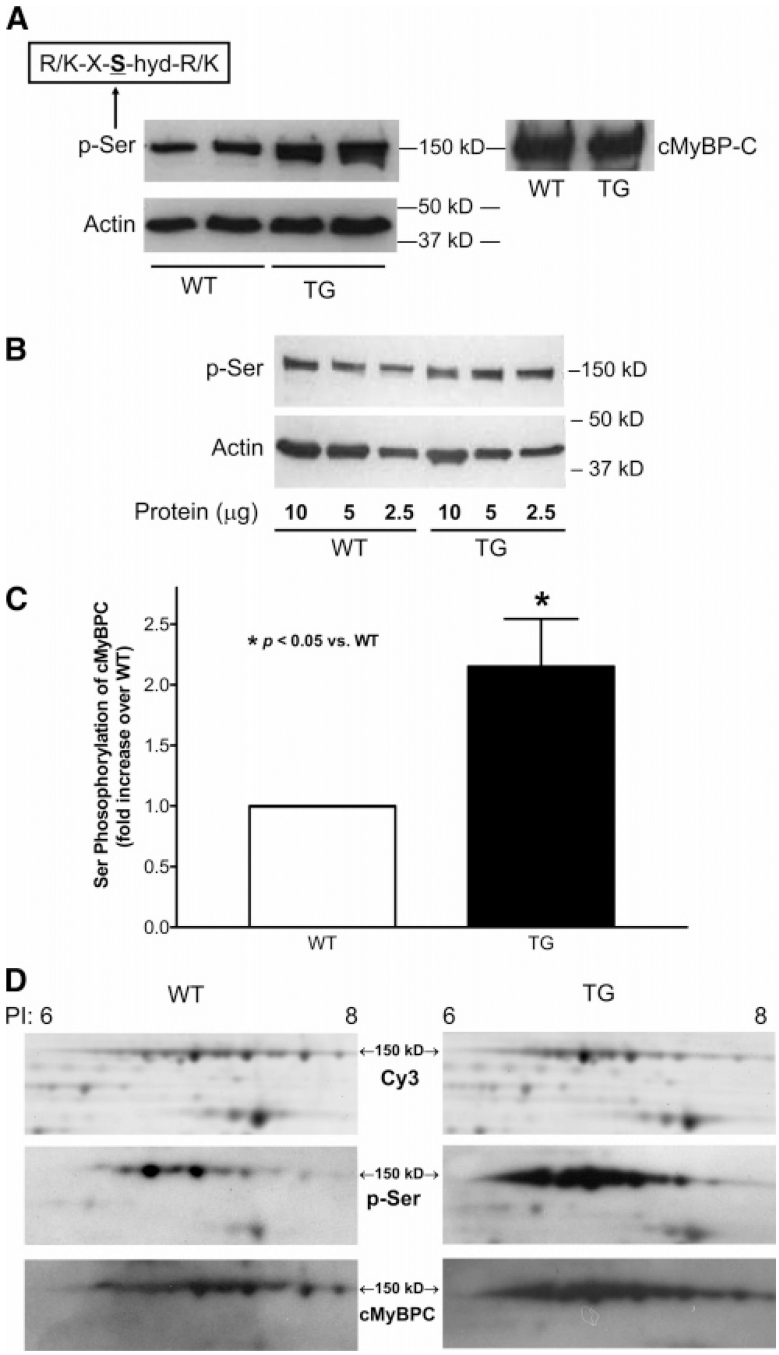

Figure 1.

Western blotting on the ventricular myofibrillar proteins extracted from 12-month-old WT or TG mouse hearts for Ser phosphorylation. (A) Representative result of SDS–PAGE gel electrophoresis and Western blotting using p-SerPKC antibody. Ten micrograms of sample was loaded in each lane. Membrane was stripped and then reprobed for actin (42 kDa) as control for loading. Cardiac MyBPC was identified at 150 kDa by Western blotting using anti-cMyBPC antibody. (B) Western blotting on serially diluted sample proteins using p-SerPKC antibody. The result confirmed the finding in panel A. (C) Quantitative densitometry analysis on the results from the Western blotting using p-SerPKC antibody; *n*

= 5 (from five pairs of hearts). The density of the p-Ser of cMyBPC was normalized to that of the stripped and reprobed actin in the same sample. The data were then presented as fold increase in Ser phosphorylation of cMyBPC over that of the WT hearts. The paired, one-tailed *t*-test was used for statistical analysis. $p < 0.05$, statistically significant. (D) 2D gel analysis on the Ser phosphorylation of cMyBPC. Equal amounts of the samples from WT or TG hearts (50 μg from each) were labeled with Cy3 and underwent 2D gel electrophoresis on separate gels as shown in the first row. The proteins were transferred simultaneously to the same PVDF membrane, and Western blotting was performed first using p-SerPKC antibody (second row). Cardiac MyBPC was detected at 150 kDa. The membrane was then stripped and reblotted for cMyBPC (third row). $n = 2$.

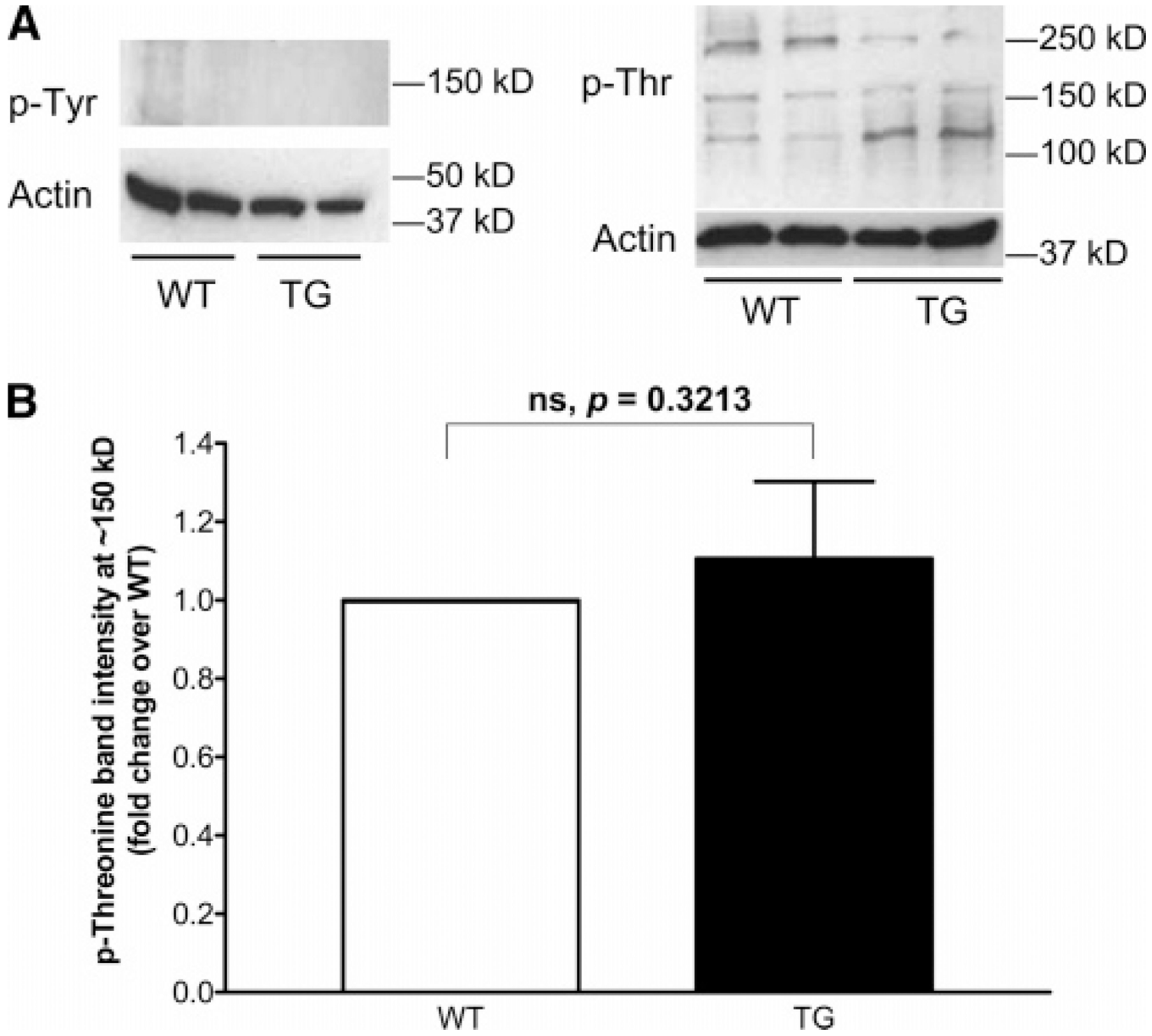

Figure 2.

Western blotting of the ventricular myofibrillar proteins extracted from 12-month-old WT or TG mouse hearts for Tyr or Thr phosphorylation using the p-Tyr antibody or the p-Thr antibody. Five micrograms of sample was loaded in each lane; Western blotting for actin was used as control for loading. (A) Representative results of Western blotting for Tyr and Thr phosphorylation. (B) Quantitative densitometry analysis of the Western blot results using p-Thr antibody; $n = 3$ (from three pairs of hearts). The density of the p-Thr at ~150 kDa was normalized to that of the stripped and reprobed actin in the same sample. The data were then presented as fold change in Thr phosphorylation over that of the WT hearts. The paired, one-tailed *t*-test was used for statistical analysis. $p = 0.3213$; ns, not significant.

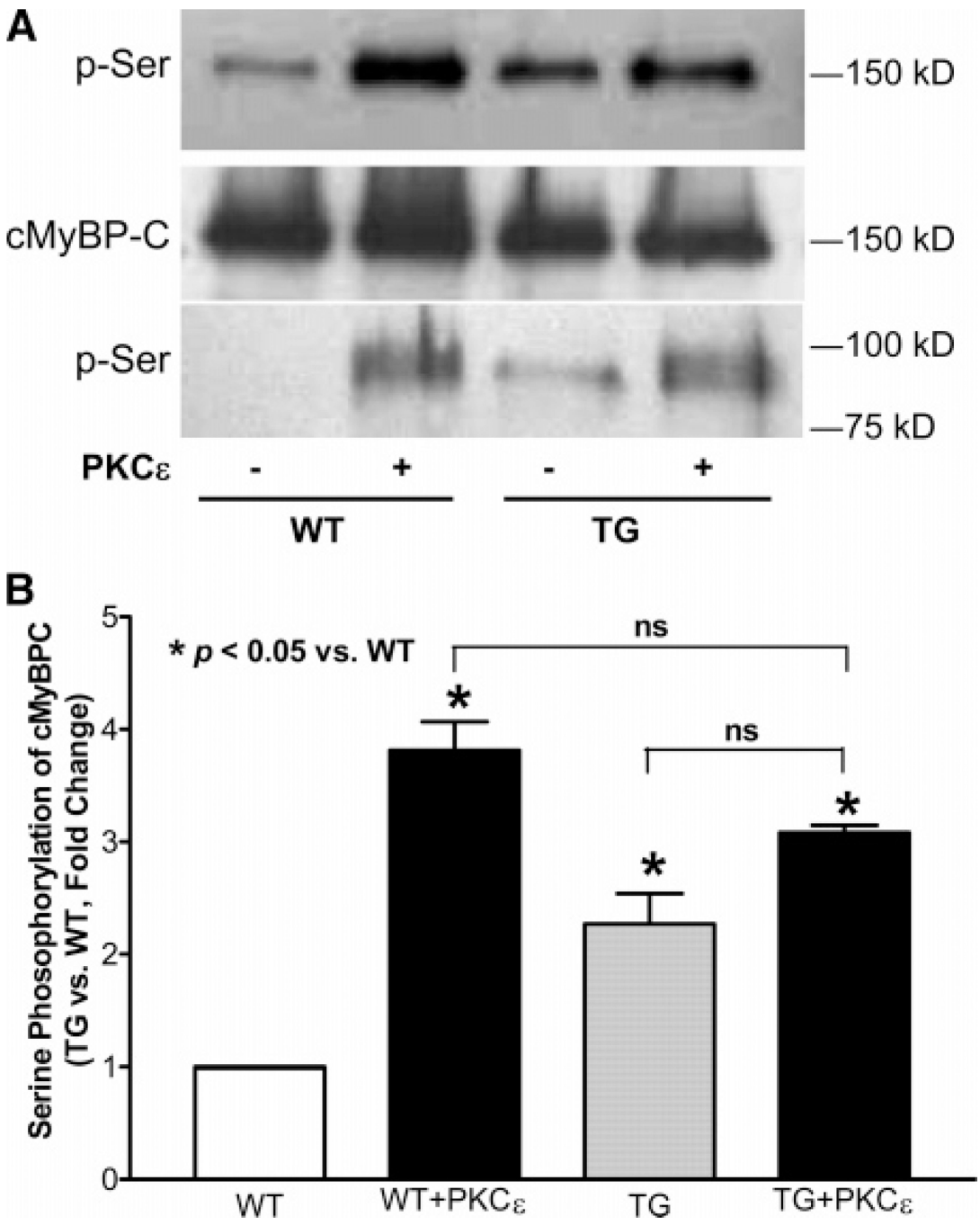

Figure 3.

In vitro PKCε assay on purified cardiac myofibrillar proteins from 12-month-old WT and TG mice followed by Western blotting using p-SerPKC antibody. The membrane was then stripped and blotted for cMyBPC. An unknown phosphoprotein identified by the p-SerPKC antibody at ~90 kDa was shown as a positive control. $n = 3$. (A) Representative result from one pair of mice. Five micrograms of sample was loaded in each lane. (B) Quantitative densitometry analysis. The data were presented as fold increase in Ser phosphorylation of cMyBPC over that of the untreated WT hearts (WT group). One-way ANOVA followed by Bonferroni's multiple comparison test was used for statistical analysis between the different groups. $p < 0.05$, statistically significant; ns, not significant.

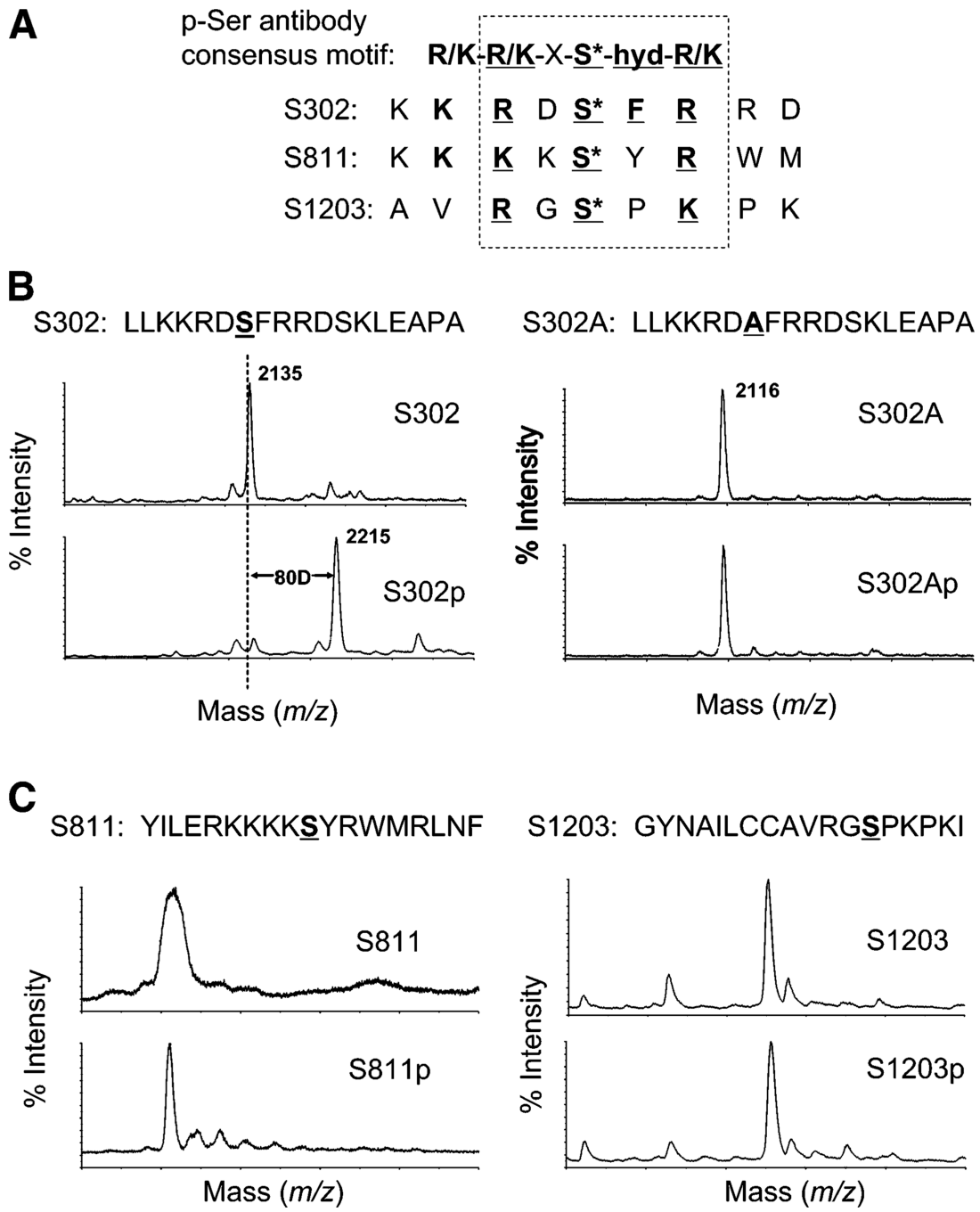

Figure 4.

Identification of phosphorylation on the PKCε-treated synthetic cMyBPC peptides. (A) The three motifs on the mouse cMyBPC coding region identified by NetPhos analysis are identical (S302) or similar (S811 and S1203) to the consensus motif for the anti-p-SerPKC antibody. (B) MS on S302 and S302A peptides with or without PKCε kinase treatment. MS identified only one phosphorylated site on S302 peptide, which was abolished after mutating the Ser302 to an Ala in the S302A peptide, indicating that the phosphorylated Ser in the S302 peptide is Ser302; *n* = 4. (C) MS on S811 and S1203 peptides with or without PKCε

kinase treatment. The study failed to identify any phosphorylation residue in either peptide after PKCε treatment; $n = 4$. p, with PKCε treatment.

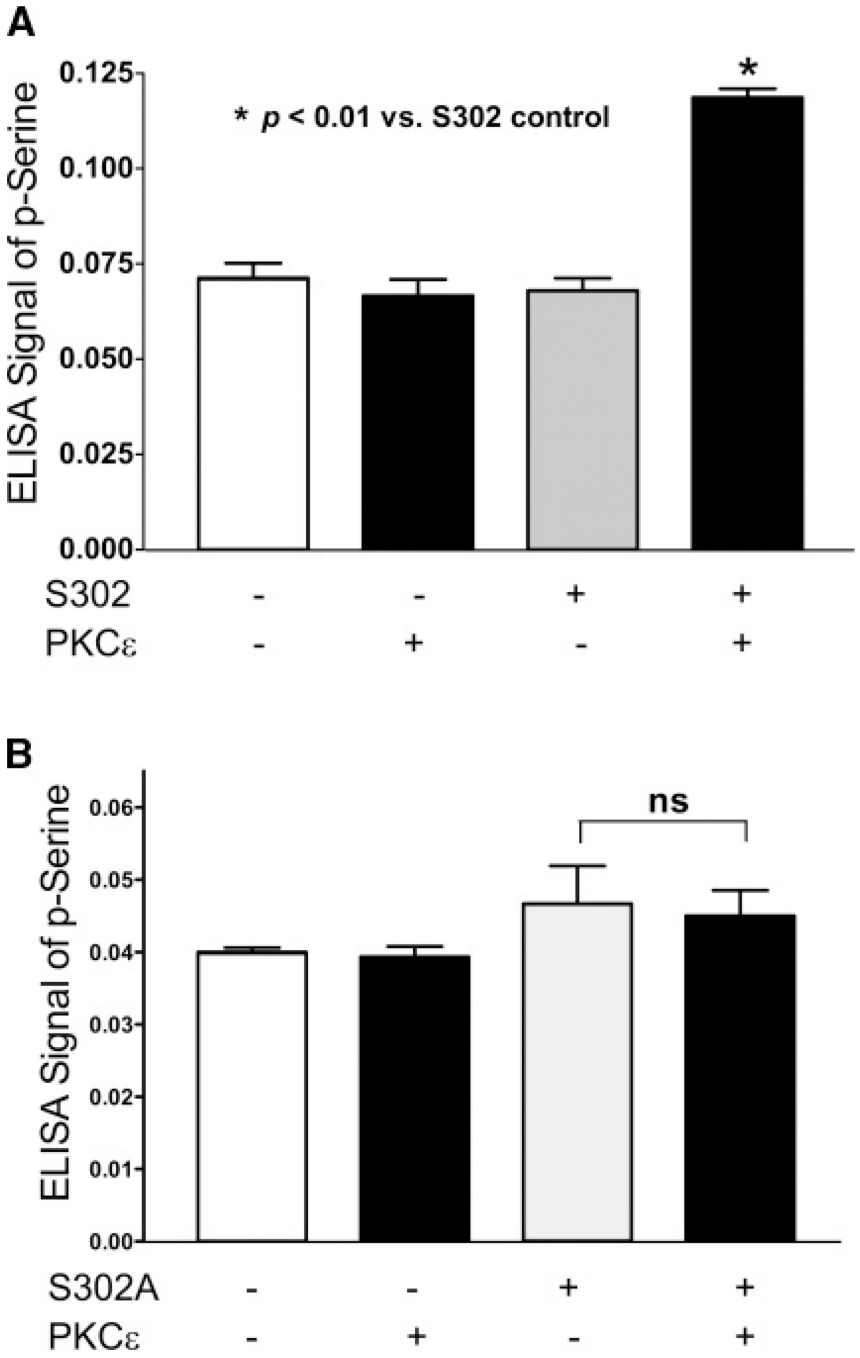

Figure 5.

ELISA to identify the phosphorylated Ser residue on the synthetic peptides after in vitro PKCε treatment. (A) ELISA on S302 peptide using p-SerPKC antibody; 80D, 80 daltons; *p*, with PKCε treatment. (B) ELISA on S302A peptide using p-SerPKC antibody. Data were pooled from three experiments ($n = 9$). The paired, one-tailed *t*-test was used for statistical analysis; $p < 0.01$, statistically significant; ns, not significant.

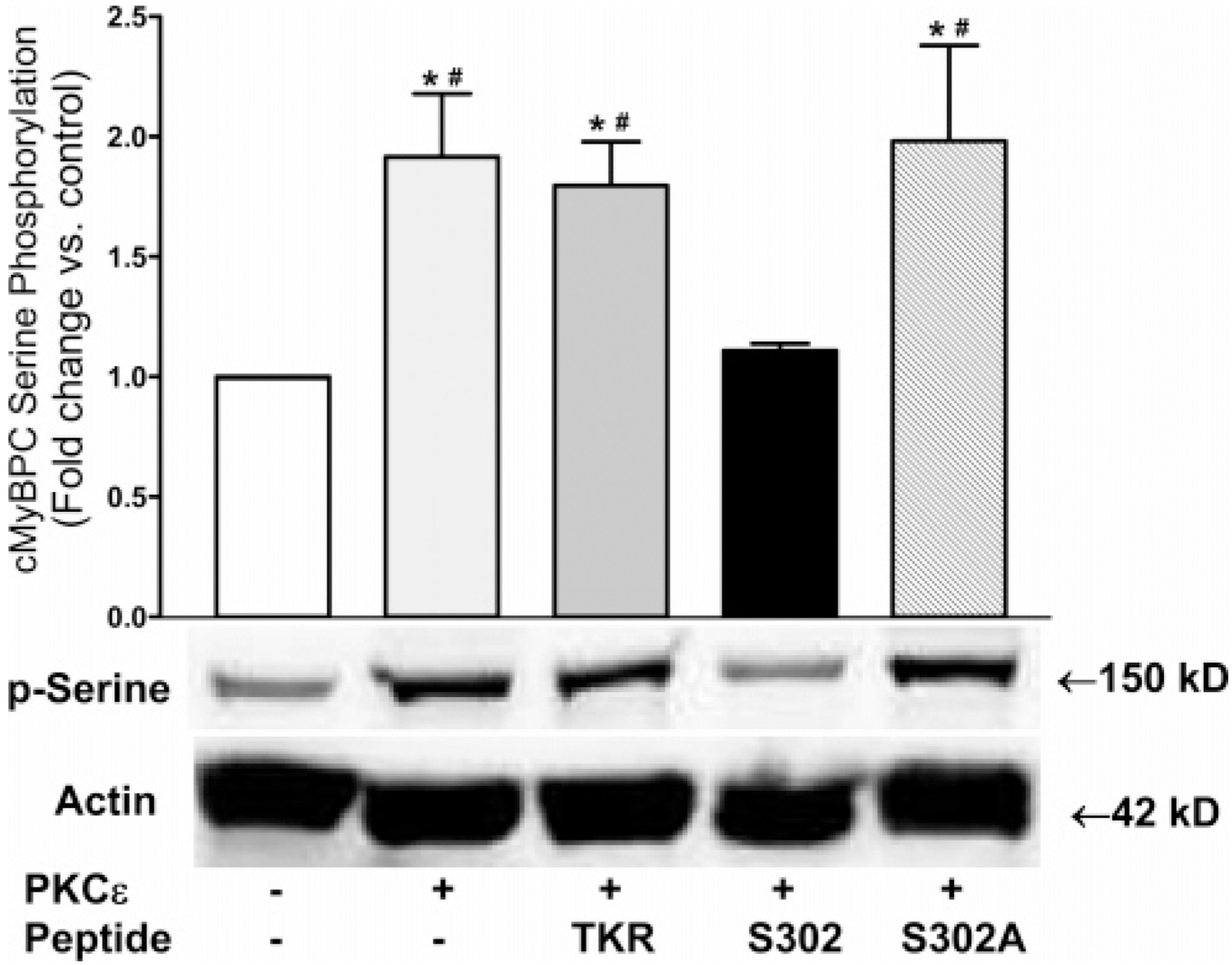

Figure 6.

In vitro PKCε competition kinase assays on intact mouse cMyBPC and the synthetic peptides followed by Western blotting using p-SerPKC antibody. Cardiac myofilaments isolated from 12-month-old WT mice were used for in vitro PKCε kinase assay. Equal amounts (5 μg) of myofilament samples were incubated with PKCε at 30 °C for 30 min in the presence of 0.1 mM synthetic peptide of either S302, the mutant S302A, or a structurally unrelated control peptide TKR (TKRKDEECSTTEHPYKKPYM) before being subjected to Western blot for detection of p-serine or actin. Actin-normalized p-serine density was analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. Data were from three independent experiments and are shown as the fold changes of the PKCε-untreated group. *, $p < 0.05$ compared to the control group; #, $p < 0.05$ compared to the group of PKCε + peptide S30.

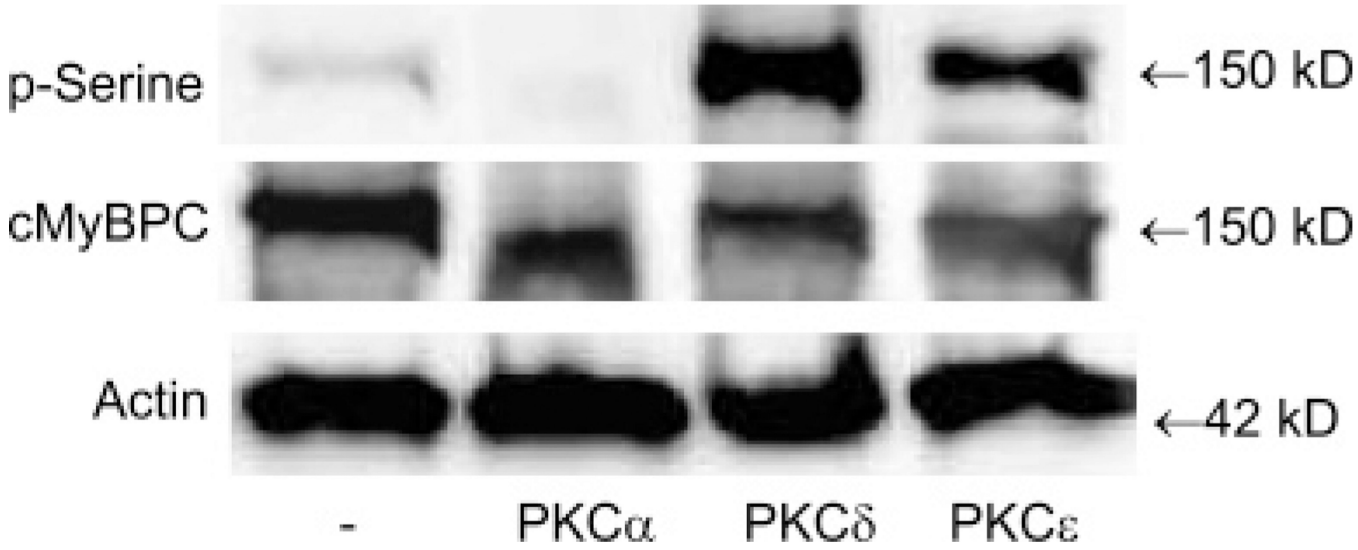

Figure 7.

In vitro PKC assay on purified cardiac myofibrillar proteins from 12-month-old WT mice followed by Western blotting. A representative Western blot result of three independent experiments was shown. Five micrograms of sample was incubated with or without an equal amount of PKCα, PKCδ, or PKCε at 30 °C for 20 min. In order to activate PKCα, the PKCα assay buffer contained 200 μM $CaCl_2$. The p-Ser and actin were first detected by Western blotting. The membrane was then stripped and reprobed for cMyBPC.