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Point mutations in the tri-helix bundle of the M-domain of cardiac myosin binding protein-C influence systolic duration and delay cardiac relaxation

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Abstract

Cardiac myosin binding protein-C (cMyBP-C) is an essential regulatory protein required for proper systolic contraction and diastolic relaxation. We previously showed that N'-terminal domains of cMyBP-C stimulate contraction by binding to actin and activating the thin filament *in vitro*. In principle, thin filament activating effects of cMyBP-C could influence contraction and relaxation rates, or augment force amplitude *in vivo*. cMyBP-C binding to actin could also contribute to an internal load that slows muscle shortening velocity as previously hypothesized. However, the functional significance of cMyBP-C binding to actin has not yet been established *in vivo*.

We previously identified an actin binding site in the regulatory M-domain of cMyBP-C and described two missense mutations that either increased (L348P) or decreased (E330K) binding affinity of recombinant cMyBP-C N'-terminal domains for actin *in vitro*. Here we created transgenic mice with either the L348P or E330K mutations to determine the functional significance of cMyBP-C binding to actin *in vivo*. Results showed that enhanced binding of cMyBP-C to actin in L348P-Tg mice prolonged the time to end-systole and slowed relaxation rates. Reduced interactions between cMyBP-C and actin in E330K-Tg mice had the opposite effect and significantly shortened the duration of ejection. Neither mouse model displayed overt

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systolic dysfunction, but L348P-Tg mice showed diastolic dysfunction presumably resulting from delayed relaxation.

We conclude that cMyBP-C binding to actin contributes to sustained thin filament activation at the end of systole and during isovolumetric relaxation. These results provide the first functional evidence that cMyBP-C interactions with actin influence cardiac function *in vivo*.

Keywords

Heart; Cardiac myosin binding protein C; Actin; Transgenic mice; Cardiac function

1. Introduction

Cardiac myosin binding protein-C (cMyBP-C) is a sarcomeric protein that is essential for regulating force and speed of cardiac muscle contraction and relaxation [1–4]. Loss of cMyBP-C in knockout mice shortens the time course of systole, accelerates cross-bridge kinetics and increases myocyte shortening velocity and power output [3, 5]. Collectively, these observations initially suggested that cMyBP-C functions as a “brake” on contraction by exerting inhibitory effects that slow cross-bridge kinetics and the time course of ventricular stiffening and relaxation [2, 3, 5]. However, it is now clear that in addition to its inhibitory effects, cMyBP-C can also potentially activate actomyosin interactions [6, 7]. For instance, the N'-terminal domains of cMyBP-C (e.g., C0-p/a-C1-M-C2, Figure 1) exert dual inhibitory and activating effects on actin-activated ATPase rates, filament sliding speed in motility assays, and force generation in permeabilized myocytes [6–10].

The strong activating effects of cMyBP-C are attributed to the ability of cMyBP-C N'-terminal domains to bind actin and displace tropomyosin toward its open state on the thin filament [7, 10]. Because movement of tropomyosin can occur even in the absence of Ca^{2+} , direct interactions of N'-terminal domains of cMyBP-C with the thin filament may constitute an independent mechanism to activate, augment, or prolong contraction, for instance at the end of systole when intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{in}}$) is declining [7, 10–12]. Conversely, inhibitory effects of cMyBP-C can be explained because binding sites for some cMyBP-C domains on actin overlap with binding sites for myosin [13, 14]. cMyBP-C could thus sterically compete with cross-bridge formation potentially speeding relaxation. Finally, by cross-linking thick and thin filaments, cMyBP-C could also act as a drag force that slows muscle shortening velocity [15]. However, despite overwhelming evidence that cMyBP-C N'-terminal domains bind to actin and modulate actomyosin interactions *in vitro* [7, 13, 16], the functional significance of cMyBP-C binding to actin to cardiac contraction *in vivo* is still unknown.

The primary obstacle to addressing the physiological significance of cMyBP-C interactions with actin for cardiac function has been the challenge of finding a way to selectively modulate cMyBP-C binding to actin *in vivo*. Here we address this challenge using two transgenic mouse models, each with a single missense mutation in the M-domain of cMyBP-C that we had previously shown to either increase (L348P) or decrease (E330K) binding affinity of cMyBP-C N'-terminal domains for actin (Figure 1) [17]. Both the L348P and the

E330K mutation were first identified as sequence variants in patients with hypertrophic cardiomyopathy (L352P and E334K in the human sequence) [18–20]. Although the pathologic significance of the mutations in people is uncertain, both mutations are located within the highly conserved tri-helix bundle of the regulatory M-domain of cMyBP-C [21]. Our *in vitro* studies further support altered function due to the mutations because the L348P mutation increased the activating and inhibitory effects of N'-terminal domains of cMyBP-C on force in permeabilized myocytes, while the E330K mutation diminished their functional effects [17]. In addition, the L348P mutation enhanced the ability of N'-terminal domains to shift tropomyosin further toward its open position on the thin filament [22]. Taken together, these data strongly suggest that the three-helix bundle of cMyBP-C exerts functional effects by interacting with actin. We therefore hypothesized that transgenic expression of the L348P or E330K mutations would reveal contractile properties affected by cMyBP-C interactions with actin during cardiac contraction *in vivo*.

Results show that the L348P mutation significantly extended the duration of systole, slowed cardiac relaxation, and slowed myocyte shortening velocity, whereas the E330K mutation had opposite effects. These data provide the first functional evidence that binding of the M-domain to actin contributes to cMyBP-C effects on contraction *in vivo*, and suggest a role for cMyBP-C in the relative duration of systole and diastole.

2. Materials and Methods

Additional details are given in the online Supplemental Materials.

2.1 L348P and E330K transgenic mice

Site-directed mutagenesis was used to create the L348P or E330K mutation in a wild-type murine cMyBP-C cDNA transgenic expression vector (Figure 1) [23]. Expression of the transgenic cMyBP-C is driven by an α MHC promotor to target expression in cardiomyocytes and the transgenic protein has a Myc-tag at the N'-terminus to allow detection of the transgenic cMyBP-C. The wild-type cMyBP-C vector was a generous gift from Drs. Richard Moss and Patricia Powers at the University of Wisconsin, Madison. Pronuclear injections with linearized vector in FVB/NJ mice were performed by the Mouse Biology Program at UC Davis (L348P) and the GEMM core at the University of Arizona (E330K). Multiple founder lines carrying each mutation were obtained.

Individual founder lines with identical mutations showed qualitatively similar effects of the mutation on cardiac function as measured by echocardiography (not shown). Therefore, progeny from a single founder line for each mutation was used in this study. *In vivo* characterization of cardiac function by echocardiograms and pressure-volume recordings were done in male mice (12–15 weeks). Protein expression and *ex vivo* force development in permeabilized cardiomyocytes was determined in 8–16 week old mice from both sexes. Mice whose hearts were used for force measurements and analysis of cMyBP-C expression were given propranolol in their drinking water (0.5–1 g/L) 4–7 days prior to euthanasia to prevent excess phosphorylation when euthanized [24, 25].

All experiments used sex and age matched mice. Non-transgenic (NTg) littermates for each line were used as controls. Treatment of all animals was in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals using protocols approved by the Institutional Animal Care and Use Committees at the University of California, Davis and the University of Arizona.

2.2 Protein quantification

Left ventricles (LV) were homogenized and proteins were separated by gel electrophoresis (N= 4–8 per group). Total cMyBP-C expression was quantified by western blot using an anti-cMyBP-C antibody [26], with an anti- α -actinin antibody (Sigma-Aldrich A7811) used as a loading control. Transgenic protein expression was detected using an anti-Myc-tag antibody (Cell Signaling Technology 2276) normalized to total cMyBP-C expression. The relative amount of transgenic L348P or E330K protein to endogenous wild-type cMyBP-C was calculated by normalizing Myc-tag-to-cMyBP-C ratios relative to a mouse model expressing exclusively transgenic wild-type cMyBP-C (WT-Tg, Figure 2A, C) [23]. Phosphorylation of cMyBP-C, cardiac troponin I (cTnI), cardiac troponin T (cTnT) and regulatory myosin light chain (MLC2) was assessed with ProQ Diamond stain (Molecular Probes).

2.3 Immunocytochemistry

To determine (transgenic) cMyBP-C localization, LV cardiomyocytes were permeabilized with 1% Triton X-100 and stained with rabbit anti-cMyBP-C polyclonal antibody [26] and mouse anti-Myc-tag monoclonal antibody (Cell Signaling Technology 2276). Cardiomyocytes were imaged using a DeltaVision Deconvolution microscope with an Olympus 100X/ 1.40, Plan Apo, IX70 objective. Image stacks were deconvolved using softWoRx® version 1.1.0 (GE Healthcare). The images were false colored in Photoshop CS 3.1 (Adobe) with cMyBP-C shown in green, and Myc-tag in red.

2.4 Hemodynamic characterization of cardiac function

Cardiac function was determined by echocardiography and by intra-cardiac catheterization to measure pressure-volume (PV) relationships. Echocardiography was performed on mice (N= 12–23 per group) mildly sedated with 1–3% isoflurane as described previously [28]. PV recordings were made in 8–17 mice per group by inserting a pressure-volume sensing catheter (Transonic Scisense) into the LV through the cardiac apex using the technique by Pacher *et al.* [27]. Pressure and volume were simultaneously recorded at rest and during temporal occlusion of the inferior vena cava to yield end-systolic and end-diastolic pressure-volume relationships. Normalized time-varying left ventricular elastance ($E_N(t)$) was calculated from PV relationships where $E_N(t) = P(t)/(V(t)-V_0)$ and V_0 is the volume intercept of the end-systolic pressure-volume relationship. Normalized time-varying elastance was then expressed relative to peak elastance (E_{es}). All hemodynamic parameters and abbreviations are given in Supplementary materials Table S1.

2.5 Force measurements in permeabilized myocytes

Permeabilized multicellular myocyte preparations were prepared and measurements performed as previously described [17]. Force measurements were determined in separate experiments for L348P-Tg and E330K-Tg myocytes and results from each were compared to their respective NTg littermates as controls. 4–5 mice per group and 2–4 preparations per mouse were used for all experiments. Force-calcium relationships were determined by measuring steady-state force at concentrations of free Ca^{2+} varying between pCa 6.2 to pCa 4.5 as previously described [17, 28]. Shortening velocity and power output were measured with a series of force clamps ranging from 0.5 – 90% of maximal force using the method of MacDonald *et al.* [29].

2.6 Statistics

All values represent Mean $\pm$ SEM. For force measurements, unpaired t-tests were used to compare Tg and NTg values. Comparisons between multiple groups were made by one-way ANOVA followed by Tukey's post-hoc test.

3. Results

3.1 Transgenic L348P- and E330K- cMyBP-C is expressed and incorporated properly into sarcomeres

Western blots of LV homogenates from L348P-Tg, E330K-Tg and NTg hearts (Figure 2A) were probed with antibodies to cMyBP-C and the Myc-tag to quantify cMyBP-C expression in transgenic mice. Results showed that the total amount of cMyBP-C was not different in hearts from L348P-Tg, E330K-Tg and NTg mice, and was not different compared to mice that expressed solely transgenic wild type cMyBP-C (WT-Tg) on a cMyBP-C knockout background [23] (Figure 2B). In L348P-Tg mice $39 \pm 4\%$ of total cMyBP-C expressed was mutant L348P-Tg cMyBP-C and E330K-Tg mice expressed $44 \pm 2\%$ mutant E330K-Tg cMyBP-C (Figure 2C) determined by comparison to WT-Tg mice that express only transgenic cMyBP-C (100%).

Proper localization of the L348P or E330K transgenic cMyBP-C in sarcomeres was confirmed by immunostaining cardiomyocytes (Figure 2D). Staining with an antibody to cMyBP-C (green) resulted in a doublet pattern in L348P-Tg, E330K-Tg and NTg tissue as expected for localization of cMyBP-C in each half of the A-band [30, 31]. As expected, immunostaining with the anti-Myc-tag antibody (red) was only visible in myocytes from L348P-Tg and E330K-Tg mice (Figure 2D). Importantly, staining with antibodies against both the Myc-tag and cMyBP-C overlapped completely (merge, yellow), indicating that transgenic L348P and E330K cMyBP-C was localized to the same position as cMyBP-C in transgenic cardiomyocytes.

Phosphorylation of sarcomeric proteins cMyBP-C, cTnI, cTnT, and MLC2 was assessed by ProQ Diamond stain. Results showed that phosphorylation of cMyBP-C and cTnI was significantly reduced in L348P-Tg hearts (Figure S1A, B). Phosphorylation of cMyBP-C and cTnI was also reduced in E330K mice, but only cMyBP-C phosphorylation was

significantly lower (Figure S1A, B). Phosphorylation of cTnT and MLC2 did not differ significantly between NTg, L348P-Tg or E330K-Tg mice (Figure S1C,D).

3.2 Cardiac remodeling in L348P-Tg mice but not in E330K mice

Hearts from 12–16 week-old mice were excised and examined for effects of L348P-Tg or E330K-Tg expression on whole heart morphology. Figure 2E displays a representative heart for each mouse model (NTg, L348P-Tg and E330K-Tg). Neither of the transgenic mouse lines showed overt cardiac hypertrophy with heart weight-to-body weight ratios comparable to NTg hearts (4.47 ± 0.18 , N=25 and 3.92 ± 0.09 , N=9 in L348P-Tg and E330K-Tg mice versus 4.40 ± 0.12 , N=31 in NTg). However, left atria (LA) of L348P-Tg mice were markedly enlarged and LA-to-body weight ratios were ~4.5x greater in L348P-Tg (0.87 ± 0.15 , N=23) compared to NTg (0.20 ± 0.06 , N=22) and E330K-Tg (0.16 ± 0.02 , N=9) mice (Figure 2F). Left ventricle (LV)-to-body weight ratios were slightly but significantly reduced in L348P-Tg mice (2.42 ± 0.06 , N=25 in L348P-Tg, versus 2.77 ± 0.04 , N=31 and 2.65 ± 0.07 , N=9 in NTg and E330K-Tg mice). Echocardiography using 2D short axis and parasternal long axis images of L348P-Tg hearts confirmed the dramatically enlarged LA *in vivo* in L348P-Tg mice (Figure S2) consistent with appearance on gross dissection. Morphometric analysis of LV dimensions by M-mode echocardiography confirmed smaller diastolic LV internal diameter (LVID) in L348P-Tg mice, possibly due to enlargement of the left ventricular peripheral wall. (Table S1, S2). E330K-Tg mice also had smaller diastolic LVID, but their wall thickness and LV/BW was normal.

3.3 Diastolic dysfunction in L348P-Tg mice

Cardiac function was evaluated by non-invasive echocardiography, and by simultaneously recording pressure (P) and volume (V) with a catheter placed in the LV. Echocardiography measurements showed preserved systolic contraction in both L348P-Tg and E330K-Tg mice, as evident from fractional shortening (FS) and ejection fraction (EF) being comparable to NTg mice (Table 1). Stroke volume (SV) and cardiac output (CO) determined by echocardiography both were decreased in L348P-Tg and E330K-Tg mice, possibly due to smaller LV size especially in L348P mice. However, when LV volume was measured directly by PV catheterization, SV and CO output were not different in L348P-Tg and E330K-Tg mice compared to NTg (Table 2).

In contrast to systolic function, diastolic relaxation was significantly impaired in L348P-Tg hearts. Doppler echo imaging revealed longer isovolumetric relaxation times (Table 1), and slower movement of the mitral valve annulus during both early (e') and late (a') phases of diastole. Mitral valve deceleration time was also longer in L348P-Tg than in NTg mice (Table 1). This apparent slower relaxation of L348P-Tg hearts was accompanied by significantly slower speed of blood inflow into the LV during early diastole (E) and particularly during late diastole (A) (Table 1, Figure S3), indicating impaired cardiac filling. Pressure-volume analysis demonstrated significant reduction of the maximal rate of pressure decay ($-dp/dt$) in L348P-Tg and tau, the exponential decay of left ventricular pressure during isovolumetric relaxation, was significantly increased in L348P-Tg compared to NTg mice (Table 2). In addition, end-diastolic pressure was significantly increased in L348P-Tg mice,

suggesting incomplete relaxation. By contrast, E330K-Tg mice showed no evidence of diastolic impairment (Tables 1 and 2).

3.4 Opposing shift in time course of normalized elastance in L348P-Tg and E330K-Tg mouse hearts

We next analyzed the time course of left ventricular stiffening during the cardiac cycle (*i.e.*, normalized time-varying elastance ($E_N(t)$) from PV recordings (Figure 3A, B). Results showed that initial time course of myocardial stiffening (elastance) during isovolumetric contraction was similar between L348P-Tg, E330K-Tg and NTg hearts. Both the time to onset-of-ejection (t_{oe}) and the relative elastance at onset-of-ejection (E_{oe}/E_{es}) were comparable between groups (Figure 3C, D, Table 2). The maximal elastance at end-systole (E_{es}) was also not significantly different between L348P-Tg, E330K-Tg and NTg hearts (Figure 3F, Table 2). However, the time to end-systole (t_{es}) was significantly delayed in L348P-Tg mice compared to NTg mice (Figure 3E), leading to a prolonged duration of ejection in L348P-Tg mice. In addition, the time course of elastance decay was significantly prolonged in L348P-Tg mice (Figure 3B). Delayed onset of relaxation and slower relaxation are likely to both contribute to the impaired diastolic function observed in the L348P-Tg mice. By contrast, t_{es} was significantly decreased in E330K-Tg mice (Figure 3B, E, Table 2) indicating a reduced duration of the ejection phase of systole in E330K-Tg mice.

3.5 Slowed shortening velocity and reduced power output in L348P-Tg myocytes

To determine whether transgenic expression of mutant L348P or E330K affected myofilament force development we measured force development in permeabilized cardiomyocytes from L348P-Tg and E330K-Tg mice in separate sets of experiments and compared each to their NTg littermates. Myofilament Ca^{2+} sensitivity of tension as measured by pCa_{50} (the pCa value at which force is half-maximal) was comparable in cardiomyocytes from L348P-Tg and NTg mice (5.99 ± 0.03 , $N=5$ versus 5.92 ± 0.02 , $N=5$, respectively) and between E330K-Tg and NTg mice (5.81 ± 0.04 , $N=4$ versus 5.82 ± 0.02 , $N=4$, respectively) (Figure 4A, B). Maximal Ca^{2+} activated force was significantly reduced by ~40% in L348P-Tg myocytes compared to NTg myocytes (13.1 ± 2.5 , $N=5$ versus 23.8 ± 2.3 mN/mm², $N=5$, respectively; Figure 4C). There was a trend toward decreased maximal Ca^{2+} activated force in E330K-Tg myocytes compared to NTg controls (24.8 ± 2.7 , $N=4$ versus 33.6 ± 2.6 mN/mm², $N=4$, respectively; Figure 4D), but the difference was not statistically significant. Passive force at pCa 9.0 tended to be higher in L348P-Tg than in NTg cardiomyocytes, but differences did not reach statistical significance (NTg: 1.5 ± 0.3 mN/mm², $N=5$ versus L348P-Tg: 2.3 ± 0.4 mN/mm², $N=5$, respectively). Cardiomyocytes from E330K-Tg mice had a significantly lower passive force compared to NTg mice (1.9 ± 0.2 mN/mm², $N=4$ versus 3.2 ± 0.5 mN/mm², $N=4$, respectively).

To assess cross-bridge cycling kinetics we first subjected myocytes to a release and re-stretch maneuver (k_{tr}) [8]. However, k_{tr} values could not be accurately determined in L348P-Tg myocytes due to high residual forces (P_{resid}) that remained after the release and re-stretch maneuver (Figure 4E). P_{resid} in L348P-Tg myocytes approached 75% of steady state force, compared to ~40% in NTg myocytes. P_{resid} in E330K-Tg myocytes was also slightly higher compared to NTg myocytes (Figure 4F). We next measured maximal shortening velocity to

assess cross-bridge kinetics using force clamps. Results showed that shortening velocities were slower at all relative loads up to 50% of $F_{\max}$ in L348P-Tg myocytes compared to NTg myocytes (Figure 5A). Power output (i.e., force * velocity) was also reduced at all levels up to 70% of $F_{\max}$ due in part to the slower shortening velocities and reduced maximal force (Figure 5B). By contrast, in E330K-Tg mice both shortening velocity and power tended to be increased over the same range of relative loads. (Figure 5A, B).

4. Discussion

The goal of this study was to probe the functional significance of cMyBP-C interactions with actin on cardiac function *in vivo*. We hypothesized that binding of cMyBP-C to actin would activate the thin filament and that thin filament activation would accelerate activation and/or slow relaxation. To test our hypothesis, we created two transgenic mouse models that each express cMyBP-C with a single missense mutation that either increases (L348P) or decreases (E330K) cMyBP-C binding affinity for actin [17].

Results demonstrate that altered binding of cMyBP-C to actin using these two point mutations changes the time course of cardiac contraction and relaxation, especially the duration of the ejection phase of systole. Plots of the time course of normalized elastance ($E_N(t)$) showed that reduced affinity of cMyBP-C to actin in E330K-Tg mice shortened the time to end-systole (t_{es}) (Figure 3B, E), while t_{es} was prolonged in L348P-Tg mice with increased cMyBP-C binding to actin. In addition, the L348P mutation caused a pronounced slowing of relaxation throughout the isovolumetric phase of diastole. These results are consistent with a role for cMyBP-C in determining the time course of cardiac stiffening as initially described in cMyBP-C knockout mice where loss of cMyBP-C shortened the overall time course of elastance and decreased the time to end-systole [5, 32]. Our results extend these findings by suggesting that the conserved tri-helix bundle in the M-domain (where the L348P and E330K mutations occur) and its binding to actin contribute to effects of cMyBP-C on the timing of cardiac contractility.

A straightforward mechanism to account for shifted time courses in the L348P-Tg and E330K-Tg mice is that binding of the tri-helix bundle to actin maintains tropomyosin in its open state following Ca^{2+} activation. Consistent with this idea, the L348P mutation enhanced movement of tropomyosin on the thin filament to a position similar to that induced by both Ca^{2+} and myosin cross-bridges [22]. Alternatively, binding of the M-domain to actin could modulate Ca^{2+} dissociation from troponin C in a manner analogous to how strongly bound cross-bridges or NEM-S1 slow Ca^{2+} dissociation [33, 34]. In either scenario the activating effects of the tri-helix bundle of cMyBP-C may act to offset shortening induced deactivation of the thin filament as $[Ca^{2+}]_{in}$ is decreasing, thereby slowing relaxation and prolonging systolic ejection. The effect may be important in matching stroke volume to heart rate or to changes in afterload because Nagayama *et al.* [32] showed that the normal increase in t_{es} in response to increased heart rate was blunted in mice deficient in cMyBP-C, as was the normal decrease in relaxation rates in response to increased afterload. Those authors further showed that the normalized time course of elastance was not changed following treatment with a β -adrenergic agonist suggesting that the changes occur through a

mechanism that is independent from other effects of cMyBP-C to increase cardiac contractility via phosphorylation.

Aside from prolonged activation of the thin filament through direct interactions with thin filament regulatory proteins, cMyBP-C binding to actin could potentially influence contraction by cross-linking thick and thin filaments [14]. cMyBP-C could thus create a force that opposes filament sliding that slows sarcomere shortening and limits power output as observed in cardiomyocytes from L348P-Tg mice (Figure 5B). L348P-Tg mice also showed reduced maximal rate of pressure development during isovolumetric contraction ($+dP/dt$, Table 2) which could be a consequence of slower sarcomere shortening. The high residual force remaining after the release and re-stretch maneuver (k_{tr}) in the L348P-Tg myocytes (Figure 4E) may also be consistent with enhanced cross-linking between the thick and thin filaments by allowing detached cross bridges to quickly reattach [35]. The reduced maximal force development in L348P-Tg myocytes (Figure 4C) could possibly be a consequence of cMyBP-C binding to actin in locations where it competes with cross-bridges [36], thus limiting the amount of cross bridges contributing to force development. However, arguing against this possibility we also saw a trend for reduced force development in E330K-Tg cardiomyocytes (Figure 4D). Therefore reduced maximal force development may be a secondary effect as often seen in failing hearts [37, 38] rather than a direct consequence of altered cMyBP-C binding to actin. Consistent with this we also observed decreased phosphorylation of cMyBP-C and cTnI in L348P-Tg and E330K-Tg mice, which may be secondary to reduced β -adrenergic signaling.

It is notable that overall systolic function was preserved in L348P hearts despite reduced shortening velocity, power output and maximal force in L348P-Tg myocytes. One possibility for this apparent discrepancy is that maximal force in permeabilized myocytes is measured at saturating Ca^{2+} concentrations that far exceed $[Ca^{2+}]_{in}$ during systolic contraction [39]. Notably, the complexity of cMyBP-C interactions with both actin and myosin filaments makes it likely that cMyBP-C exerts a combination of effects that together optimize cardiac contraction under the varying conditions of load and Ca^{2+} activation during a single heartbeat.

The L348P and E330K mutations were first identified as rare sequence variants in patients with HCM [18, 20]. Detailed family pedigrees and patient phenotypes are not published for patients carrying the L352P variant (human sequence numbering), but our results showing that cardiac contraction is altered L348P-Tg mice supports the pathogenicity of this mutation. The E334K variant (human sequence numbering) is a more common sequence variant [20] that has been reported in numerous patients. While the frequency of its occurrence suggests that the variant may be more benign, some patients exhibited cardiac dilation and dysfunction [20]. While the E330K-Tg mice had no overt signs of cardiac dysfunction or remodeling under baseline conditions, the observed increase in shortening velocity and power output is consistent with a “hypercontractile” phenotype often found in HCM [40, 41]. It is possible that a more overt phenotype will emerge in the E330K mice when they are stressed. Alternatively, Bahrudin *et al.* [20] found that cMyBP-C with the E334K mutation was more rapidly degraded by the ubiquitin-proteasome system (UPS) suggesting that cardiac dysfunction in human patients could potentially be caused by

haploinsufficiency or by UPS impairment. Although these factors may contribute to human disease as a result of the E334K mutation, it is unlikely that they contribute to the phenotypes of transgenic E330K mice which showed normal cMyBP-C expression (Figure 2B) and because the transgenic E330K cDNA may escape normal mRNA and protein quality control mechanisms.

In summary, we show for the first time using two novel transgenic mouse models that thin filament activating effects observed for the N'-terminal domains of cMyBP-C *in vitro* [6–9] are relevant to cardiac function *in vivo*. Specifically, we demonstrate that L348P-Tg expression caused a marked slowing of relaxation and lengthened the time of systolic ejection, presumably reflecting the enhanced ability of cMyBP-C N'-terminal domains with the L348P mutation to bind actin and maintain the thin filament in an active state [7, 22]. Importantly, thin filament activation by L348P-Tg appeared to preferentially slow diastolic relaxation but did not significantly affect activation during isovolumetric contraction. The latter suggests that the ability of cMyBP-C to activate the thin filament through its tri-helix bundle may be most relevant following the initial rise in $[Ca^{2+}]_{in}$ when the thin filament is already open (Figure 6). This is consistent with observations that cMyBP-C binding to myofibril I-bands was increased in the presence of Ca^{2+} [42] and that activating effects of the C1 domain required the presence of a small amount of activating Ca^{2+} [10]. Thus, the activating effects of cMyBP-C N'-terminal domains on the thin filament may influence cardiac function by sustaining contraction during the late stages of systole as sarcomeres shorten and $[Ca^{2+}]_{in}$ begins to decline.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Cardiac myosin binding protein C binding to actin affects cardiac function *in vivo*.
- cMyBP-C regulates timing of systole through binding to actin.
- Increased binding of cMyBP-C to actin slowed cardiac relaxation.
- Increased cMyBP-C binding to actin caused diastolic dysfunction in mice.

Figure 1. Schematic of L348P and E330K transgenic cMyBP-C

Cardiac mutant cMyBP-C expression was driven by an α -MHC promoter. An N'-terminal Myc-tag was inserted upstream of the full-length mouse cMyBP-C cDNA to distinguish transgenic protein.

Figure 3. Pressure-volume analysis of cardiac function in L348P-Tg, E330K-Tg and NTg mice
A) Representative PV loops of NTg, L348P-Tg and E330K-Tg mice during temporal occlusion of the inferior vena cava. **B)** The time course of normalized elastance ($E_n(t)$) was prolonged in L348P-Tg mice. E330K-Tg mice showed a shorter time to end-systole (t_{es}) compared to NTg mice. **C)** Time of onset of ejection (t_{oe}) was not different between groups, nor was the relative elastance (E_{oe}/E_{es}) at T_{oe} (**D**). **E)** t_{es} was later in L348P-Tg mice and earlier in E330K-Tg mice. **F)** Absolute maximal elastance was not significantly different in NTg, L348P-Tg and E330K-Tg mice. * $P < 0.05$ vs NTg, # $P < 0.05$ vs E330K-Tg. NTg: N=17, L348P-Tg, N=8, and E330K-Tg, N=8.

Figure 4. Effects of transgenic L348P and E330K expression on force-pCa relationships and residual force in permeabilized myocytes

Relative force plotted against pCa for (A) L348P-Tg and matching NTg littermate control myocytes and (B) E330K-Tg and matching NTg littermate control myocytes. Ca^{2+} -sensitivity of tension was not significantly different in Tg myocytes compared to control myocytes. (C) Maximal Ca^{2+} activated force at pCa 4.5 was significantly lower in L348P-Tg myocytes. (D) Maximal Ca^{2+} activated force was lower, but not significantly in E330K-Tg myocytes. (E) Residual force (F_{resid}) at pCa 4.5 was significantly higher in L348P-Tg than in NTg cardiomyocytes. (F) In E330K-Tg cardiomyocytes F_{resid} was somewhat larger than NTg control myocytes. *P < 0.05 vs NTg. L348P-Tg vs NTg: N=5 in each group. E330K-Tg vs NTg: N=4 in each group.

Figure 5. Shortening velocity and power output in myocytes from NTg, L348P-Tg, and E330K-Tg mice

A) Loaded shortening velocity and B) calculated power (force * velocity) were normalized to maximal shortening velocity or power as measured in NTg cardiomyocytes and plotted for L348P-Tg (N=5), E330K-Tg (N=4), and NTg control (N=8) myocytes. Results showed that loaded shortening velocity and power output were significantly decreased in L348P-Tg myocytes compared to NTg myocytes up to 50% and 70% of F_{max}, respectively. Cardiomyocytes from E330K-Tg mice showed greater shortening velocity and power output compared to NTg cardiomyocytes up to 30% and 70% of F_{max}, respectively.

Figure 6. Model for cMyBP-C interactions with thin filaments during the cardiac cycle

(I) During the filling phase of diastole the thin filament (actin in blue and troponin-tropomyosin in yellow) is not activated. cMyBP-C (green) and the myosin cross bridges (XB, purple) are not bound to actin. Upon activation of the thin filament by Ca^{2+} (orange hexagons) cross bridges and cMyBP-C bind to actin. (II) Pressure and elastance rise during isovolumetric contraction. (III) During the ejection phase of the cardiac cycle the actin filament slides past myosin and the cardiomyocytes contract and shorten. cMyBP-C binding to actin during this time sustains thin filament activation as sarcomeres shorten and $[\text{Ca}^{2+}]_i$ declines. This allows cMyBP-C to influence the duration of ejection as seen in L348P and E330K Tg hearts. cMyBP-C binding to actin may also oppose filament sliding limiting cardiac contraction. (IV) During isovolumetric relaxation the thin filament becomes fully inactivated as $[\text{Ca}^{2+}]_i$ and cross-bridges unbind from the thin filament. cMyBP-C may also unbind in response to sarcomere lengthening. However, if cMyBP-C abnormally remains bound to actin during this time relaxation could be slowed as observed in L348P-Tg mice.

Table 1

Echocardiographic analysis of cardiac function in L348P-Tg, E330K-Tg and NTg mice.

	NTg	L348P-Tg	E330K-Tg
HR (bpm)	505 ± 9	491 ± 9	498 ± 5
Systolic function	(N=23)	(N=21)	(N=12)
SV (ml)	47.4 ± 1.2	42.4 ± 1.0 *	42.6 ± 1.9
EF (%)	63.5 ± 1.4	64.2 ± 1.8	65.8 ± 1.2
FS (%)	34.4 ± 1.0	34.9 ± 1.3	35.8 ± 0.9
CO (mL/min)	23.9 ± 0.7	20.7 ± 0.5 *	21.2 ± 1.0 *
Diastolic function	(N=24)	(N=13–19)	(N=12)
E (mm/s)	965.0 ± 23.8	584.2 ± 26.5 *,#	990.0 ± 22.8
A (mm/s)	448.8 ± 18.6	167.9 ± 17.8 *,#	410.7 ± 20.6
E/A	2.2 ± 0.1	3.9 ± 0.4 *,#	2.5 ± 0.1
IVCT (ms)	9.7 ± 0.2	9.3 ± 0.4	8.9 ± 0.2
IVRT (ms)	9.9 ± 0.2	18.5 ± 0.6 *,#	9.3 ± 0.3
e' (mm/s)	30.9 ± 1.3	17.2 ± 1.5 *,#	30.8 ± 1.0
a' (mm/s)	16.6 ± 0.8	9.1 ± 1.7 *,#	14.9 ± 0.9
e'/a'	1.9 ± 0.1	2.1 ± 0.2	2.1 ± 0.1
E/e'	32.3 ± 1.4	36.8 ± 2.9	32.5 ± 1.2
MV decel time (ms)	22.4 ± 0.9	29.3 ± 1.5 *,#	22.5 ± 0.9

For abbreviations, see Table S1.

*
p<0.05 versus NTg,#
p<0.05 versus E330K-Tg.

Table 2

Pressure-volume analysis of cardiac function in L348P-Tg, E330K-Tg and NTg mice.

	NTg (N=17)	L348P-Tg (N=8)	E330K-Tg (N=8)
HR (BPM)	481 ± 8	458 ± 8	488 ± 10
Systolic function			
ESP (mmHg)	88.2 ± 1.9	80.9 ± 2.5	80.7 ± 2.5
ESV (μL)	30.6 ± 2.0	24.4 ± 2.9 #	37.5 ± 3.9
SV (μL)	49.3 ± 2.1	49.7 ± 4.4	44.3 ± 4.1
CO (mL/min)	23.6 ± 0.9	22.7 ± 2.0	21.6 ± 2.2
EF (%)	61.8 ± 2.2	67.0 ± 4.1 #	54.3 ± 3.4
+dP/dt (mmHg/s)	9,115 ± 319	7,704 ± 281 *	8,658 ± 418
ESPVR	4.3 ± 0.4	2.7 ± 0.5	3.7 ± 0.6
V ₀	3.1 ± 3.3	-15.6 ± 3.6 *,#	9.4 ± 5.6
Diastolic function			
EDP (mmHg)	4.0 ± 0.4	10.1 ± 1.0 *,#	4.2 ± 0.7
EDV (μL)	79.9 ± 2.2	74.1 ± 4.5	81.8 ± 5.1
-dP/dt (mmHg/s)	-7,418 ± 209	-3,630 ± 165 *,#	-7,238 ± 381
Tau Glantz (ms)	14.0 ± 0.8	39.1 ± 2.6 *,#	14.8 ± 1.3
EDPVR	0.07 ± 0.01	0.13 ± 0.02 *,#	0.06 ± 0.01
Normalized time-varying elastance (E_N(t))			
t _{oe} (ms)	16.9 ± 0.5	16.7 ± 1.0	16.6 ± 0.4
t _{es} (ms)	48.6 ± 1.2	55.8 ± 1.4 *,#	42.9 ± 1.2 *
E _{oe} /E _{es}	0.37 ± 0.03	0.45 ± 0.04	0.40 ± 0.03
E _{es} (mmHg/μl)	3.8 ± 0.4	2.4 ± 0.3	3.5 ± 0.5

For abbreviations, see Table S1.

*
p<0.05 versus NTg,#
p<0.05 versus E330K-Tg.