A Gain-of-Function Mutation in the M-domain of Cardiac Myosin-binding Protein-C Increases Binding to Actin* [S]

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Kristina L. Bezold, Justin F. Shaffer1, Jaskiran K. Khosa, Elaine R. Hoye, and Samantha P. Harris2

From the University of California, Davis, California 95618

The M-domain is the major regulatory subunit of cardiac myosin-binding protein-C (cMyBP-C) that modulates actin and myosin interactions to influence muscle contraction. However, the precise mechanism(s) and the specific residues involved in mediating the functional effects of the M-domain are not fully understood. Positively charged residues adjacent to phosphorylation sites in the M-domain are thought to be critical for effects of cMyBP-C on cross-bridge interactions by mediating electrostatic binding with myosin S2 and/or actin. However, recent structural studies revealed that highly conserved sequences downstream of the phosphorylation sites form a compact tri-helix bundle. Here we used site-directed mutagenesis to probe the functional significance of charged residues adjacent to the phosphorylation sites and conserved residues within the tri-helix bundle. Results confirm that charged residues adjacent to phosphorylation sites and residues within the tri-helix bundle are important for mediating effects of the M-domain on contraction. In addition, four missense variants within the tri-helix bundle that are associated with human hypertrophic cardiomyopathy caused either loss-of-function or gain-of-function effects on force. Importantly, the effects of the gain-of-function variant, L348P, increased the affinity of the M-domain for actin.

In the heart, the influence of cardiac (c) MyBP-C on contraction is controlled by phosphorylation of a cluster of 3–4 cardiac-specific sites located within a unique regulatory motif referred to as the “M-domain.” The M-domain is positioned in the N-terminal half of the molecule between two folded immunoglobulin (Ig) domains (i.e. C1 and C2, Fig. 1A). Sequences within the M-domain are targets for a growing list of protein kinases including PKA, PKC, PKD, p90 S6, and CamKII (1–5).

When cMyBP-C is phosphorylated it speeds rates of actomyosin interactions leading to enhanced cardiac contractility following inotropic stimuli (6–8) such as the “fight or flight” response that occurs upon β-adrenergic stimulation. Reduced levels of cMyBP-C phosphorylation are associated with heart failure (9–12), whereas constitutive phosphorylation of cMyBP-C is protective against ischemia-reperfusion damage (13).

Despite the significance of cMyBP-C to cardiac function, however, the underlying mechanism(s) by which the M-domain and its phosphorylation affect cross-bridge cycling remain only partly understood. Putative mechanisms have focused on the idea that electrostatic charge interactions at or surrounding the phosphorylatable serine control binding interactions that mediate functional effects. For instance, positively charged arginine and lysine residues adjacent to the phosphorylatable serines have been proposed to mediate electrostatic binding interactions with myosin S2 (14) or actin (15, 16) and these interactions are reduced or abolished upon introduction of negatively charged phosphate groups upon phosphorylation (17, 18). However, simple charge screening and on/off binding interactions may not be sufficient to fully describe the functional effects of the M-domain in sarcomeres because the ability of N-terminal domains of cMyBP-C to influence force in permeabilized trabeculae was reduced but not eliminated by phosphorylation suggesting that additional sequences are involved in mediating the effects of cMyBP-C on cross-bridge interactions (19).

Additional clues into the mechanisms by which the M-domain affects contraction have come from recent structural studies showing that the structure of the M-domain consists of dynamic or disordered regions including the phosphorylation consensus sites (20, 21), whereas the C terminus of the M-domain consists of three α-helices arranged in a compact bundle.
Notably, sequences encoding this tri-helix bundle are highly conserved across all isoforms of MyBP-C, suggesting that it mediates a common or conserved function in different muscle types. Consistent with this idea, skeletal muscle isoforms of myosin-binding protein C can exert similar effects as cardiac MyBP-C on actomyosin interactions in motility assays (22).

The current experiments were designed to investigate the functional significance of the tri-helix bundle and to determine whether positively charged residues adjacent to cardiac-specific phosphorylation sites are sufficient for the functional effects of the M-domain. Results confirm that residues adjacent to phosphorylation sites contribute to the function of the M-domain, but that residues within the tri-helix bundle that are common to all isoforms of MyBP-C also contribute to the ability of the M-domain to influence actomyosin interactions.

**EXPERIMENTAL PROCEDURES**

Recombinant Protein Expression and Purification—Recombinant proteins were expressed and purified using murine cDNA sequences as described previously (23, 24). For single and paired amino acid substitutions, site-directed mutagenesis of wild-type C1C2 (domains C1-M-C2) was performed using the QuikChange II Site-directed Mutagenesis kit (Stratagene) with primers designed using the Stratagene primer design software. For deletion of helix 3 from C1C2 (H3), the target sequence was deleted from the C1C2 sequence using the Infusion system (Clontech) and amplified with the High Fidelity Phusion Enzyme (Finnzymes, ThermoFisher). The H3 sequence was then treated with DpnI to digest methylated parental DNA and gel purified prior to ligation into pQE2 vector using the Infusion system.

For protein expression, cDNA vectors were used to transform M15 cells (Qiagen) and expression was induced with isopropyl 1-thio-β-D-galactopyranoside. Expressed proteins were purified under non-denaturing conditions using a nickel-nitrilotriacetic acid affinity column (Thermo) according to the manufacturer’s directions. After column elution in a buffer containing (in mM): 250 imidazole, 25 Tris-HCl, 200 NaCl, 1 2-mercaptoethanol, 0.1 PMSF, and 1 g/ml of Pepstatin A, pH 7.5, fractions containing expressed protein were pooled and exchanged into the desired buffers using protein desalting spin columns (Thermo) for mechanical force measurements or biochemical assays. Protein concentrations were measured using a Nanodrop spectrophotometer (Thermo) using extinction coefficients calculated (ExPASy-ProtParam) from primary protein sequences.

Protein Phosphorylation—Expressed proteins were treated with recombinant protein kinase A (PKA, Sigma) as previously described (17). Following PKA treatment proteins were again purified using a nickel-nitrilotriacetic acid affinity column (Thermo), according to the manufacturer’s directions. After column elution in a buffer containing (in mM): 250 imidazole, 25 Tris-HCl, 200 NaCl, 1 2-mercaptoethanol, 0.1 PMSF, and 1 μg/ml of Pepstatin A, pH 7.5), fractions containing expressed protein were pooled and exchanged into the desired buffers using protein desalting spin columns (Thermo) for mechanical force measurements or biochemical assays. Protein concentrations were measured using a Nanodrop spectrophotometer (Thermo) using extinction coefficients calculated (ExPASy-ProtParam) from primary protein sequences.

Cosedimentation Binding Assays—Cosedimentation binding assays were performed and analyzed as described previously (17). Briefly, recombinant cMyBP-C proteins were dialyzed against a cosedimentation buffer containing (in mM): 20 imidazole, 180 KCl, 1 MgCl₂, 1 EGTA, 1 DTT, pH 7.4. F-actin from bovine heart was prepared from ether powder as described (26).
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and was stored prior to use in a buffer containing (in mM) 50 KCl, 1 MgCl₂, 2 Tris-HCl, pH 8.0, 0.2 CaCl₂, 0.5 2-mercaptoethanol, 1 ATP, and 0.02% sodium azide. Recombinant cMyBP-C proteins (1–30 μM final concentration) were combined with F-actin, ATP, and DTT to achieve final concentrations of 5 μM, 1 mM, and 1 mM, respectively, in a final volume of 50 μl of cosedimentation buffer.

Animals and Preparation of Permeabilized Trabeculae Preparation—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 Committee at University of California, Davis. Male Sprague-Dawley rats (200–250 g) were euthanized by intraperitoneal injection of sodium pentobarbital. Hearts were then rapidly excised and right ventricles were dissected in a Ringer’s solution containing (in mM): 100 NaCl, 24 NaHCO₃, 2.5 KCl, 1 MgSO₄, 7H₂O, 1 Na₂HPO₄, and 1 CaCl₂. Trabeculae were permeabilized in situ by incubation of splayed ventricles overnight at 4°C in a relaxing solution containing 50% glycerol and 1% Triton X-100 (Sigma). Individual trabeculae were then dissected free from ventricular walls, pinned to the bottom of a sylgard-coated Petri dish, and stored in glycerinated relaxing solution at −20°C for up to 1 week before use.

Solutions—Relaxing and activating solutions were prepared using a custom software package as previously described (23, 27). Solutions contained (in mM): 15 phosphocreatine, 15 EGTA, >40 MOPS, 1 free Mg²⁺, 135 Na⁺ + K⁺, 1 dithiothreitol, 250 units ml⁻¹ creatine kinase, 5 ATP. pH was adjusted to 7.0 at 15°C and total ionic strength was 0.18 m. Solutions with Ca²⁺ concentrations of pCa 9.0 and pCa 4.5 (where pCa = −log([Ca²⁺])) were created by adding appropriate amounts of CaCl₂. Activating solutions with Ca²⁺ concentrations between pCa 6.4 and 5.2 were made by mixing pCa 9.0 and 4.5 solutions. A pre-activating solution was made by replacing EGTA with HDTA in the pCa 9.0 solution.

Mechanical Force Measurements—Force measurements in permeabilized trabeculae were performed as described previously (23). Permeabilized trabeculae were mounted between a force transducer (model 403A, Aurora Scientific Inc.) and a torque motor (model 312-C, Aurora Scientific Inc.) for rapid adjustments of fiber length. After securing each end of the trabecula, the entire stainless steel plate (model 802D, Aurora Scientific Inc.) containing the experimental wells and the trabecula was placed on the stage of an inverted microscope (Olympus IX71) fitted with a 12 megapixel digital camera (Olympus DP70) for visualization of the trabecula and measurement of fiber dimensions and sarcomere length. Sarcomere length was adjusted to ~2.25 μm in relaxing solution and monitored throughout the course of an experiment (model 604A software, Aurora Scientific Inc.).

Force was measured during steady-state contraction in Ca²⁺ buffers containing submaximal or maximal Ca²⁺ (pCa 6.2–4.5) at 15°C. Prior to each activation, the trabecula was incubated in a pre-activating solution with reduced Ca²⁺ buffering for 2 min. Maximal force measurements were obtained at saturating Ca²⁺ (i.e. in a pCa 4.5 solution) at the beginning and the end of each series of submaximal force measurements. Force-pCa data were plotted by expressing submaximal force (P) at each pCa as a fraction of maximal force measured at pCa 4.5 (P₉₀), which is P/P₉₀, where P/P₉₀ = P₉₀. The rate of force redevelopment (kᵣ) following a 20% release and re-stretch including a 1-ms 5% overshoot was measured during each activation after steady-state force had reached a plateau value in a given pCa solution as previously described (27).

Force Measurements with Recombinant MyBP-C Proteins—Each trabecula served as its own control and force-pCa curves were obtained before and after exposure to recombinant proteins. Force-pCa relationships were first obtained as described above in the absence of added proteins and then individual trabeculae were incubated sequentially in a relaxing solution (pCa 9.0, 15 min) and a pre-activating solution (2 min) that contained the desired concentration of recombinant protein. Trabeculae were then transferred briefly (1–2 min) to Ca²⁺-containing solutions for force measurements before returning to the relaxing (pCa 9.0) solution. In most cases recombinant proteins were omitted from Ca²⁺ activating solutions because measured forces were not different with or without added protein (not shown), consistent with previous results demonstrating negligible washout of proteins during the short period of Ca²⁺ activation (23).

Statistics—All values represent mean ± S.D. unless otherwise noted. Comparisons between groups or before and after treatments were made by one-way analysis of variance followed by Bonferroni pairwise comparisons between groups.

RESULTS

Positively Charged Residues Adjacent to Phosphorylation Sites Are Required for Effects of C1C2—Basic residues upstream of phosphorylation consensus sites have been implicated in the functional effects of the M-domain by mediating electrostatic binding interactions with myosin S2 (14) and/or actin (15, 16). In particular, Arg²⁶⁶, Arg²⁷⁰, and Arg²⁷¹ at the N terminus of the M-domain were necessary for stereospecific binding interactions with F-actin and for the ability of a 29-kDa N-terminal fragment of cMyBP-C to inhibit sliding velocity in an in vitro motility assay (16). To determine whether these residues are also required for the functional effects of the M-domain on force in permeabilized trabeculae, we mutated these residues, alone or in pairs, to alanines and assayed their effects on the ability of a recombinant C1C2 protein (inclusive of domains C1-M-C2) to increase Ca²⁺ sensitivity of tension and force redevelopment (kᵣ) in permeabilized trabeculae. Fig. 2 shows effects of 5 μM wild-type C1C2 on force and effects of 5 μM of a mutant C1C2 in which Arg²⁷⁰ and Arg²⁷¹ were replaced with alanines. Wild-type C1C2 significantly increased the Ca²⁺ sensitivity of tension (ΔpCa₉₀ = 0.29 ± 0.09, Fig. 2A), consistent with previous results showing that exogenous C1C2 added to either permeabilized trabeculae (23) or myocytes from cMyBP-C knock-out mice (that lack endogenous cMyBP-C) (19) caused a leftward shift of the tension-pCa relationship. However, compared with C1C2 the R270A,R27¹ mutant was much less effective and caused much less of a shift in Ca²⁺ sensitivity of tension (Fig. 2B Table 1). In addition, whereas the wild-type C1C2 protein was effective at increasing the rate of tension redevelopment, kᵣ, to near maximal rates even at low force or [Ca²⁺] (Fig. 2C), 5 μM of the R270A,R27¹A did not
significantly increase \( k_{tr} \) above control values before incubation with protein (Fig. 2D).

To determine whether these effects were unique to residues Arg270 and Arg271 adjacent to Ser273 or whether additional charged residues upstream of the other phosphorylation consensus sites also contribute to the functional effects of the M-domain, we mutated each positively charged doublet adjacent to phosphorylatable serines Ser282, Ser302, and Ser307. Results shown in Fig. 3A indicate that mutation of residues upstream of phosphorylation sites Ser282, Ser302 (i.e. R279A, R280A and K298A, K299A) significantly reduced the effects of C1C2 on Ca2+ sensitivity of tension (i.e. the mutations decreased the \( \Delta p_{Ca_{so}} \) compared with that of wild-type C1C2). However, the substitution R304A, R305A adjacent to Ser307 had negligible effect. The latter result is consistent with observations that Ser307 is phosphorylated to a lesser extent and that effects of phosphorylation are more modest compared with Ser273, Ser282, and Ser302 (16, 28, 29).

**Actin Binding Sequences in H3 Contribute to Effects of C1C2**—Because pairs of basic residues also occur outside of the cardiac phosphorylation sites we next investigated whether charged amino acids that are located toward the C terminus of the M-domain also affect function of C1C2. For instance, the lysine/arginine pair Lys346–Arg347 occurs in helix 3 (H3) of the tri-helix bundle and within a sequence proposed to be a consensus-binding site for actin (i.e. LK(R/K)XK) (21). Notably, this sequence is also part of a more extended sequence (VTDLRMLK) that bears a high degree of homology to the actin binding segment of the inhibitory peptide of troponin I (30) (Fig. 1A). As shown in Fig. 3B, replacement of Lys346–

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**TABLE 1**

Effects of C1C2 variants on tension-pCa relationship values

All proteins are 5 μM unless otherwise stated.

| Protein description | Protein (n) | \( p_{Ca_{so}} \) before protein | \( p_{Ca_{so}} \) after protein | \( \Delta p_{Ca_{so}} \) | \( n_{tr} \) before protein | \( n_{tr} \) after protein |
|---------------------|-------------|----------------------------------|--------------------------------|------------------------|---------------------------|---------------------------|
| Wild-type           | C1C2 (9)    | 5.70 ± 0.01                      | 6.00 ± 0.01*                   | 0.29 ± 0.09            | 4.82 ± 0.56               | 2.56 ± 0.07*               |
| Residues near       | R270A, R271A (6) | 5.63 ± 0.01                      | 5.72 ± 0.02*                   | 0.08 ± 0.05*           | 6.45 ± 0.36               | 4.34 ± 0.66               |
| phosphorylation sites | R279A, R280A (3) | 5.70 ± 0.01                      | 5.82 ± 0.01*                   | 0.12 ± 0.04*           | 5.84 ± 0.44               | 4.12 ± 0.32               |
| K298A, K299A (5)    | 5.63 ± 0.02                      | 5.73 ± 0.01*                   | 0.05 ± 0.06*           | 4.51 ± 0.91               | 4.57 ± 0.28               |
| Residues within H3/Tnl homology sequence | R304A, R305A (6) | 5.61 ± 0.01                      | 5.84 ± 0.01*                   | 0.24 ± 0.06            | 5.50 ± 0.64               | 2.87 ± 0.16*               |
| K346A, R347A (3)    | 5.67 ± 0.01                      | 5.76 ± 0.01*                   | 0.07 ± 0.03*           | 6.27 ± 0.70               | 4.52 ± 0.41               |
| K349A (3)           | 5.68 ± 0.01                      | 5.79 ± 0.01*                   | 0.11 ± 0.02*           | 5.25 ± 0.35               | 3.24 ± 0.15               |
| H3 (3)              | 5.70 ± 0.01                      | 5.93 ± 0.01*                   | 0.25 ± 0.02            | 6.24 ± 0.59               | 3.62 ± 0.31               |
| HCM variants        | R325Q (6) | 5.67 ± 0.01                      | 5.86 ± 0.01*                   | 0.19 ± 0.04*           | 5.21 ± 0.24               | 3.17 ± 0.18               |
| E330K (9)           | 5.72 ± 0.01                      | 5.84 ± 0.01*                   | 0.15 ± 0.07*           | 4.37 ± 0.14               | 3.55 ± 0.26               |
| V338D (4)           | 5.66 ± 0.01                      | 5.79 ± 0.01*                   | 0.13 ± 0.03*           | 4.96 ± 0.45               | 4.06 ± 0.37               |
| PKA treatment       | C1C2-P (5) | 5.73 ± 0.01                      | 5.75 ± 0.01*                   | 0.02 ± 0.02*           | 3.89 ± 0.18               | 4.04 ± 0.16               |
| L338P-P (3)         | 5.75 ± 0.01                      | 5.91 ± 0.01*                   | 0.17 ± 0.01*           | 6.75 ± 0.45               | 3.71 ± 0.13               |
| \( \Delta H3 \)     | 5.67 ± 0.01                      | 5.69 ± 0.01*                   | 0.03 ± 0.02*           | 5.83 ± 0.65               | 7.37 ± 1.10               |
| 10 AM \( \Delta H3 \) (4) | 5.67 ± 0.01                      | 5.77 ± 0.01*                   | 0.10 ± 0.02*           | 5.64 ± 0.62               | 5.80 ± 0.78               |

* Significance, \( p < 0.05 \) versus control \( p_{Ca_{so}} \) or \( n_{tr} \).
* Significance, \( p < 0.05 \) versus 5 μM C1C2.
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Figure 3. Effects of C1C2 and C1C2 mutants on Ca^{2+} sensitivity of tension. Data are plotted as the magnitude of the shift in Ca^{2+} sensitivity of tension before and after incubation with the indicated protein (ΔpCa_{50}). A, C1C2 and alanine substitution mutants that replace basic residues adjacent to each of the four phosphorylation sites, Ser273, Ser282, Ser302, and Ser307. Mutants associated with the 273, 282, and 302 sites reduced the ability of C1C2 to increase Ca^{2+} sensitivity, but the mutant associated with Ser307 did not. B, C1C2 (data re-plotted from panel A) and the indicated alanine substitutions within H3 and at residues outside of H3 (K356A,K357A). *, significance, p < 0.05 versus C1C2.

Figure 4. Effect of deletion of the H3 sequence from C1C2 (ΔH3). A, normalized force-pCa relationships before (black circles) and after (open circles) incubation of skinned trabeculae with 5 μM ΔH3. B, effect of C1C2 and ΔH3 on force in permeabilized trabeculae in the absence of activating Ca^{2+} (pCa 9.0). C1C2 activated force in the absence of Ca^{2+} at concentrations ≥10 μM, whereas ΔH3 required ≥ 30 μM. *, significance, p < 0.05 versus 0 μM.

Additional positive charges occur within the TnI homology region of H3. To determine whether these residues also affect function of C1C2, we replaced individual amino acids including R342A or K349A with alanines. As shown in Fig. 3B both individual substitutions significantly reduced the Ca^{2+} sensitizing effects of C1C2 (Fig. 3B). The magnitude of reduction caused by either individual point mutation was similar to that achieved by the charge doublet substitutions near the cardiac phosphorylation sites or the doublet Lys^{346–Arg}^{347} (Fig. 3B). However, replacing lysines 356 and 357, which lie outside of H3 near the C terminus of the M-domain did not significantly affect the ability of C1C2 to increase tension. Collectively, these results demonstrate that specific basic residues throughout the M-domain can influence the functional effects of C1C2.

H3 Enhances the Contractile Effects of C1C2—The ability of individual point mutations within the H3 sequence to affect the function of C1C2 to an extent comparable with residues adjacent to phosphorylation sites suggested that the H3 helix and/or the tri-helix bundle structure are important for mediating the functional effects of the M-domain. To determine whether H3 is essential for the effects of C1C2 in cardiac trabeculae, we next investigated whether more extensive disruption of H3 and the TnI homology region could eliminate the ability of C1C2 to enhance force at submaximal Ca^{2+} activation. Fig. 4 shows the effects of a mutant C1C2 protein in which amino acids^{340–DLRGMLKRLK}^{349} encoding the H3 helix were deleted (ΔH3).

Compared with wild-type C1C2, ΔH3 significantly reduced the Ca^{2+} sensitizing effects of the M-domain such that effects of 5 μM ΔH3 were not significantly different from control tension-pCa relationships obtained in the absence of added protein (Fig. 4A, Table 1). However, as shown in Table 1, the Ca^{2+} sensitizing effects of C1C2 could be partially recovered by increasing the concentration of ΔH3 protein > 10 μM. Importantly, the ΔH3 protein also retained the ability to activate force even in the absence of Ca^{2+} (at pCa 9.0), albeit at much higher concentrations than C1C2 (Fig. 4B). The latter results demonstrate that whereas H3 contributes to effects of C1C2 in cardiac trabeculae, sequences outside of H3 must contribute to the activating effects of C1C2 because elimination of H3 did not fully eliminate these effects.

Variants in the Tri-helix Bundle Linked to HCM Affect M-domain Function—To further investigate the functional significance of the tri-helix bundle, we next tested whether single amino acid missense variants linked to human hypertrophic cardiomyopathy (HCM) affected the activity of C1C2. To date, four missense variants were reported that occur within the three conserved helices: R322Q in helix 1 (H1), E330K and V338D in helix 2 (H2), and L348P in H3 (21, 31). Fig. 5 shows that the first three of these variants, R322Q, E330K, and V338D,
which occur within H1 and H2, significantly reduced the ability of C1C2 to increase Ca\(^{2+}\) sensitivity of tension. Because each of these variants reduced the effects of C1C2, these variants could constitute “loss-of-function” mutations if similar deficits occur in sarcomeres in vivo. However, in marked contrast to these mutations, the L348P variant located within H3 had more complex effects on force. As shown in Fig. 6A, 5 \(\mu M\) L348P significantly increased effects of C1C2 such that force was activated even in the absence of activating Ca\(^{2+}\) (i.e. at \(p_{Ca} 9.0\)). L348P also reduced maximum Ca\(^{2+}\)-activated force at saturating Ca\(^{2+}\) (\(p_{Ca} 4.5\)). Because wild-type C1C2 activates force independent of Ca\(^{2+}\) at concentrations >10 \(\mu M\) (23) and can inhibit maximum force at concentrations >10 \(\mu M\) (Fig. 6B), the L348P mutation could be considered a “gain-of-function” variant that enhances the functional effects of C1C2.

To determine whether the force enhancing effects of the L348P mutation are regulated by phosphorylation, we next treated the L348P protein with PKA (L348P-P). It was previously shown that phosphorylation of human C1C2 by PKA (C1C2-P) reduces effects of C1C2 on Ca\(^{2+}\) sensitivity (19). In agreement with this we show here that phosphorylation of murine C1C2 also reduced its ability to increase Ca\(^{2+}\) sensitivity (Fig. 7A) and \(k_e\) (Fig. 7B). Phosphorylation of the mutant L348P by PKA also reduced its ability to shift Ca\(^{2+}\) sensitivity, but to a lesser extent than C1C2-P (Fig. 7A). However, L348P-P still increased \(k_e\) to near maximal values at all levels of force similar to the effects of unphosphorylated C1C2 (Fig. 7B). Thus, phosphorylation of L348P only partially reduced its ability to increase force and the rate of tension redevelopment. Importantly, the L348P mutation did not affect the ability of PKA to phosphorylate C1C2 because intact protein and trypsin-digested proteins analyzed by mass spectroscopy (supplemental Fig. 1) demonstrated that the L348P protein was phosphorylated to a similar extent as wild-type C1C2. These data thus demonstrate that sequences remote from the cardiac-specific phosphorylation sites can influence the functional effects of the M-domain and show unequivocally that simple on/off electrostatic charge interactions cannot solely account for effects of C1C2 on force.

The L348P Mutation Increases Binding Affinity of C1C2 for Actin—To gain insight into the mechanism(s) by which mutations in the M-domain affect the functional properties of C1C2, we assessed the ability of the various C1C2 mutant proteins to interact with actin. Both the charged amino acids near phosphorylation sites and sequences in H3 have been proposed to mediate interactions with actin (15, 16, 21).

Fig. 8 and Table 2 show summary results of cosedimentation binding assays for mutants with alanine substitutions at charged residues adjacent to phosphorylation sites and in the proposed actin binding sequences of H3. Notably, none of the eight individual or paired alanine charge mutants affected the \(K_d\) for actin binding, although some of the mutants appeared to reduce the total amount of actin bound (\(B_{max}\)) consistent with electrostatic charge interactions. The HCM variant R322Q located in H1 also had no affect on actin binding. However, the H2 variants E330K and V338D significantly reduced the affinity of C1C2 for actin, as did the \(\Delta H3\) deletion (Fig. 8). In contrast to these mutations, however, the H3 mutant L348P significantly increased the apparent affinity of C1C2 for actin (i.e. reduced \(K_d\) Fig. 8). Taken together, these data support a role for H2 and H3 of the tri-helix bundle in mediating the actin binding properties of the M-domain.

**DISCUSSION**

The main conclusions from this study are that the functional effects of the M-domain of cMyBP-C cannot be accounted for solely by electrostatic charge interactions due to residues near the cardiac-specific phosphorylation sites and that effects on actin binding are mediated at least in part by other highly conserved sequences within the M-domain. Because the conserved residues are common across all isoforms of MyBP-C, results presented here have broad implication for the function of MyBP-C in different muscle types.
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FIGURE 7. Effects of PKA phosphorylation of C1C2 and L348P on Ca\(^{2+}\) sensitivity of force and rate of tension redevelopment (k\(_{tr}\)) in skinned trabeculae. A, data are shown as the magnitude of the shift in Ca\(^{2+}\) sensitivity of tension before and after incubation with the indicated protein (\(\Delta p_{Ca50}\)). Data for C1C2 are re-plotted from Fig. 3 for purposes of comparison. B, force-

k\(_{tr}\) relationship showing effects of C1C2 (black triangle), C1C2-P (dark gray boxes), and L348P-P (light gray diamonds) compared with control measurements in the absence of protein (open circles).

Conclusions from this study are based on the findings that substitutions of amino acids that are remote in primary sequence from the canonical phosphorylation sites, with either alanine residues or with other amino acid variants associated with human HCM, altered the effects of the M-domain on force and the rate of force re-development (k\(_{tr}\)). In theory, substitutions that reduce the effects of C1C2 could do so indirectly by disrupting protein secondary or tertiary structure such that phosphorylation sites were also affected (for instance, such that phosphorylation sites are no longer exposed). However, this explanation cannot account for persistence of the functional effects of C1C2 following complete phosphorylation of the M-domain (29) or for the ability of L348P to enhance force even after phosphorylation by PKA (Fig. 7A). Thus, results presented here provide strong support for a functional role of the conserved tri-helix bundle in mediating the effects of the M-domain in a manner that is distinct from the electrostatic effects exerted by phosphorylation sites. Furthermore, because HCM variants in helixes 2 and 3 also affected the actin binding affinity of the M-domain and correlated with gain- or loss-of-function effects (Figs. 5 and 8), results provide support for the idea that the actin binding properties of the M-domain are important in mediating the normal function of cMyBP-C in sarcomeres.

Functional Effects of the M-domain Involve Phosphorylation Sites as Well as Conserved Sequences within the Tri-helix Bundle—N-terminal domains of cMyBP-C, including the proline-alanine rich region, C1, and the M-domain, have all been demonstrated to profoundly affect actomyosin interactions in a wide variety of in vitro or ex vivo systems (7, 8, 23, 24, 32–38). With respect to the M-domain, previous studies focused primarily on the cardiac-specific phosphorylation sites that are present at the N terminus of the M-domain because these sites are thought to control on/off electrostatic binding interactions with myosin S2 (38) and because phosphorylation of these residues accelerate cross-bridge cycling (32, 35, 36). The first 17 amino acids of the M-domain were also found to be necessary for stereo-specific binding interactions with actin in a laser trap assay (16). The view that these residues constitute the primary functional unit of the M-domain has gained additional support from observations that a 29-kDa proteolytic fragment containing only the first 17 residues of the M-domain becomes cleaved during ischemic stress and that the 29-kDa proteolytic fragment (also referred to as a 40-kDa fragment due to its anomalous migration on SDS-PAGE) impairs cardiac contractile function and is cytotoxic to cells (39). Further support for the

![Image](image-url)

**TABLE 2**

Cosedimentation binding data for C1C2 and mutants

| Protein (n) | \(K_B\) | \(B_{max}\) |
|------------|--------|-----------|
| C1C2 (7)   | 7.4 ± 2.4 | 0.90 ± 0.13 |
| R270A,R271A (5) | 8.6 ± 1.2 | 0.70 ± 0.06* |
| R279A,R280A (4) | 5.1 ± 1.7 | 0.63 ± 0.13* |
| K298A,K299A (3) | 6.0 ± 0.8 | 0.66 ± 0.04* |
| R304A,R305A (5) | 8.1 ± 2.2 | 0.59 ± 0.05* |
| R342A (3) | 7.9 ± 2.2 | 0.59 ± 0.05* |
| K346A,K347A (3) | 10.6 ± 2.1 | 0.94 ± 0.16 |
| K349A (3) | 7.4 ± 2.4 | 0.90 ± 0.13 |
| K356A,K357A (3) | 10.9 ± 1.3 | 0.84 ± 0.06 |
| R322Q (5) | 10.9 ± 2.1 | 0.87 ± 0.15 |
| E330K (4) | 26.6 ± 5.9 | 1.26 ± 0.24* |
| V338D (4) | 21.0 ± 6.1 | 1.31 ± 0.30* |
| L348P (7) | 3.2 ± 1.4* | 1.01 ± 0.16 |
| \(\Delta H_3\) (3) | 19.3 ± 5.7* | 0.83 ± 0.12 |
| L348P-P (3) | 5.3 ± 1.7 | 0.72 ± 0.13 |

* Significance, \(p < 0.05\) versus C1C2.

![Image](image-url)

**FIGURE 8.** Actin binding affinity (\(K_D\), white bars) and molar binding ratios (\(B_{max}\), gray bars) of C1C2, HCM variants, and \(\Delta H_3\). *Inset* shows a representative cosedimentation gel. Pellets P1–P7 all contain 5 \(\mu\)M actin and increasing concentrations of C1C2 (P1 = 1 \(\mu\)M; P2 = 5 \(\mu\)M; P3 = 10 \(\mu\)M; P4 = 15 \(\mu\)M; P5 = 20 \(\mu\)M; P6 = 25 \(\mu\)M; P7 = 30 \(\mu\)M). P8 is a no actin control with 30 \(\mu\)M C1C2. * indicates significance, \(p < 0.05\) versus C1C2.

![Image](image-url)
significance of these residues has come from a variety of transgenic mouse models showing that selective mutation of individual phosphorylatable serine residues can cause disparate phenotypes from benign to the development of hypertrophy (13, 36, 40–42).

Results presented here using site-directed mutagenesis to substitute alanine residues for the naturally occurring positively charged residues within phosphorylation consensus sites confirm that basic residues immediately upstream of the phosphorylation site Ser272 contribute to the functional effects of the N terminus of cMyBP-C. We further demonstrate that basic residues upstream of Ser282 and Ser302 also contribute to the ability of C1C2 to enhance Ca2+ sensitivity of tension in permeabilized cardiac muscle (Fig. 3). However, functional effects of the M-domain were also significantly reduced by point mutations at other positively charged residues including Lys342, Lys349, and Lys346-Arg347 that are located within helix 3 (H3) of the tri-helix bundle (Table 1, Fig. 3). These results implicate residues outside of the cardiac-specific phosphorylation sites in mediating the functional effects of C1C2.

The Tri-helix Motif Encodes Actin Binding Site(s) of the M-domain of cMyBP-C—Insights into how residues remote from the cardiac phosphorylation sites affect M-domain function were provided by recent structural data from NMR studies that revealed that the M-domain consists of a relatively dynamic N terminus followed by a more structured C terminus composed of three sequential α-helices arranged in a compact and folded configuration (21) (Fig. 1B). The structure is in good agreement with predictions from atomic force microscopy and computational analyses that indicated that the M-domain contains both disordered and ordered sequences (20, 21). Interestingly, based on disorder prediction algorithms, the most ordered segment of the entire M-domain is predicted to be the sequence corresponding to helix 2 (H2). The propensity for order in this segment and its high degree of conservation across MyBP-C sequences from fish to humans suggests that it encodes a conserved binding site (for review of intrinsically disordered proteins see Ref. 43). Candidate ligands identified for the M-domain thus far include myosin S2 (14, 18, 44), actin (34, 45–47), and/or Ca2+/calmodulin (48). In support of the idea that H2 encodes a conserved ligand-binding site, the E330K and V338D variants in H2, which were identified based on their presence in humans with HCM, significantly decreased the binding affinity of C1C2 for actin (Fig. 8).

Primary sequence analysis of the tri-helix motif in the M-domain further identified a putative consensus-binding site for actin (i.e. LK(R/K)/XK) in H3 of the tri-helix bundle (21, 49). Notably, this sequence occurs as part of a more extended sequence that bears homology to the inhibitory peptide of troponin I that binds actin (30). Because deletion of this sequence in the ∆H3 mutant reduced the binding affinity of C1C2 for actin (Fig. 8), the sequence is likely to contribute to actin binding in cMyBP-C as well. However, given the sequence homology with the TnI inhibitory peptide, interactions with other thin filament proteins cannot be ruled out.

Additional evidence to implicate H3 in actin binding was provided by the L348P HCM variant because it unexpectedly enhanced actin-binding affinity (Fig. 8). This result was surpris-
data is that binding of the M-domain to actin is not necessary for its functional effects. However, the close correlation between the ability of the HCM variant L348P to enhance the effects of C1C2 on force and to increase the actin binding affinity of C1C2 strongly argues for a relationship between the two. One possibility is that binding of the M-domain to actin or to other proteins of the thin filament occurs via a two-step process such that charged residues near the phosphorylation sites are first necessary for initial low affinity electrostatic “docking” interactions that position the M-domain for subsequent higher affinity binding interactions involving the tri-helix bundle. The decrease in total amount of C1C2 bound to actin in proteins in which the basic residues had been substituted with alanines (Bmax, Table 2) is consistent with such a mechanism, as is the reduced Bmax measured for phosphorylated C1C2 (17). However, other explanations are possible and it is likely that the effects of phosphorylation encompass more than simple changes in net charge that mediate electrostatic interactions. For instance, phosphorylation often induces disorder-to-order transitions in dynamic sequences (50) and Howarth et al. (21) suggested that such transitions in the M-domain could introduce additional helical content into the sequences surrounding the phosphorylation sites. Consistent with this Michalik et al. (51) using circular dichroism and dynamic light scattering showed that the structure of the M-domain was altered by phosphorylation. Such changes in secondary structure could affect binding interactions with other sarcomeric ligands or within the M-domain itself, for example, through interactions with solvent-exposed basic residues in helix 3.

Conclusion—Results from this study establish that the effects of the M-domain of cMyBP-C to influence actomyosin interactions cannot be accounted for solely by on/off electrostatic binding interactions of residues adjacent to cardiac-specific phosphorylation sites. Instead, effects of the M-domain are mediated at least in part through additional conserved residues of the tri-helix bundle including residues in helices 2 and 3 that affect actin binding.

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