

Phosphorylation and calcium antagonistically tune myosin-binding protein C's structure and function

Michael J. Previs^{a,1}, Ji Young Mun^{b,1,2}, Arthur J. Michalek^{a,1,3}, Samantha Beck Previs^a, James Gulick^c, Jeffrey Robbins^c, David M. Warshaw^{a,4}, and Roger Craig^{b,4}

^aDepartment of Molecular Physiology and Biophysics, Cardiovascular Research Institute, University of Vermont, Burlington, VT 05405; ^bDepartment of Cell and Developmental Biology, University of Massachusetts Medical School, Worcester, MA 01655; and ^cDivision of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229

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During each heartbeat, cardiac contractility results from calcium-activated sliding of actin thin filaments toward the centers of myosin thick filaments to shorten cellular length. Cardiac myosin-binding protein C (cMyBP-C) is a component of the thick filament that appears to tune these mechanochemical interactions by its N-terminal domains transiently interacting with actin and/or the myosin S2 domain, sensitizing thin filaments to calcium and governing maximal sliding velocity. Both functional mechanisms are potentially further tunable by phosphorylation of an intrinsically disordered, extensible region of cMyBP-C's N terminus, the M-domain. Using atomic force spectroscopy, electron microscopy, and mutant protein expression, we demonstrate that phosphorylation reduced the M-domain's extensibility and shifted the conformation of the N-terminal domain from an extended structure to a compact configuration. In combination with motility assay data, these structural effects of M-domain phosphorylation suggest a mechanism for diminishing the functional potency of individual cMyBP-C molecules. Interestingly, we found that calcium levels necessary to maximally activate the thin filament mitigated the structural effects of phosphorylation by increasing M-domain extensibility and shifting the phosphorylated N-terminal fragments back to the extended state, as if unphosphorylated. Functionally, the addition of calcium to the motility assays ablated the impact of phosphorylation on maximal sliding velocities, fully restoring cMyBP-C's inhibitory capacity. We conclude that M-domain phosphorylation may have its greatest effect on tuning cMyBP-C's calcium-sensitization of thin filaments at the low calcium levels between contractions. Importantly, calcium levels at the peak of contraction would allow cMyBP-C to remain a potent contractile modulator, regardless of cMyBP-C's phosphorylation state.

muscle activation | muscle regulation | cMyBP-C | structure-function

Cardiac contractility results from the calcium-dependent sliding of actin-based thin filaments toward the centers of myosin-based thick filaments to shorten the overall length of the sarcomere, the heart's elementary contractile unit. Filament sliding is turned on and off on a beat-to-beat basis by calcium's binding to, and release from, the troponin-tropomyosin regulatory complex on the thin filament. These mechanochemical regulatory processes are fine-tuned by proteins within the thick filament, including cardiac myosin-binding protein C (cMyBP-C). Although cMyBP-C is not essential for cardiac contractility, its importance in contractile function is evidenced by mutations in the *MYBPC3* gene being a leading cause of inherited hypertrophic cardiomyopathy (1, 2). Because of the prevalence of this disease (affecting 1 in 500 people) and the potential for therapeutic intervention, much work during the last two decades has focused on defining cMyBP-C's structure and function within the sarcomere (3).

cMyBP-C has an elongated modular structure comprising 11 Ig and fibronectin type III (Fn3) domains, numbered C0–C10 from the N terminus (Fig. 1A) (4, 5). The C-terminal domain (C10) is tightly bound to the thick filament backbone, and the N-terminal domains extend radially from the thick filament (6, 7). Thus cMyBP-C's N-terminal domains are positioned to bind to neighboring actin

filaments and/or the myosin S2 domain to modulate actomyosin activity. However, consensus regarding the actual binding sites and partner(s) in vivo has not yet been reached (8). The N-terminal domains contain long polypeptide linkers between the C0–C1 and C1–C2 domains, the latter termed the “M-domain” or “motif” (Fig. 1A). The M-domain contains four highly conserved serines (S273, S282, S302, and S307; mouse sequence) in an intrinsically disordered region of its N-terminal half and a three-helix bundle (residues 317–351) in its C-terminal region (Fig. 1A) (9, 10). β -Adrenergic-stimulated phosphorylation of these serines is believed to enhance cardiac contractility (11, 12), and a high level of phosphorylation appears to be critical to normal cardiac function, whereas dephosphorylation has been associated with heart failure (13–15). Although the mechanistic role of cMyBP-C phosphorylation in vivo remains unclear, it is known to reduce the extensibility of the M-domain (10, 16), diminish the binding of cMyBP-C to actin (17, 18) and to myosin S2 (19), and to tune cMyBP-C's ability to modulate actomyosin activity in vitro (20–24).

To gain insight into the function of cMyBP-C in its native structural environment, we recently developed a total internal reflectance microscopy (TIRFM)-based assay to visualize the sliding of actin-based filaments along native cardiac thick (myosin)

Significance

Mutations in cardiac myosin-binding protein C (cMyBP-C) are the leading cause of inherited hypertrophic cardiomyopathy, demonstrating the key role that cMyBP-C plays in the heart's contractile machinery. Since its discovery >40 years ago, cardiovascular biologists have wondered how cMyBP-C's structure regulates its function. Using atomic force spectroscopy, electron microscopy, in vitro model systems of contractility, and mutant protein expression, we show how phosphorylation affects cMyBP-C's structure to tune its functions within the contractile apparatus. We also identify a novel molecular mechanism by which calcium ions that trigger each muscle contraction override phosphorylation's structural and functional impact. This intersection of posttranslational modification and calcium signaling may occur in other biological systems that rely on these pathways to control cellular processes.

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¹M.J.P., J.Y.M., and A.J.M. contributed equally to this work.

²Present address: Department of Biomedical Laboratory Science, College of Health Science, Eulji University, Seongnam-Si, Gyeonggi-Do 13135, Republic of Korea.

³Present address: Department of Mechanical and Aeronautical Engineering, Clarkson University, Potsdam, NY 13699.

⁴To whom correspondence may be addressed. Email: david.warshaw@uvm.edu or roger.craig@umassmed.edu.

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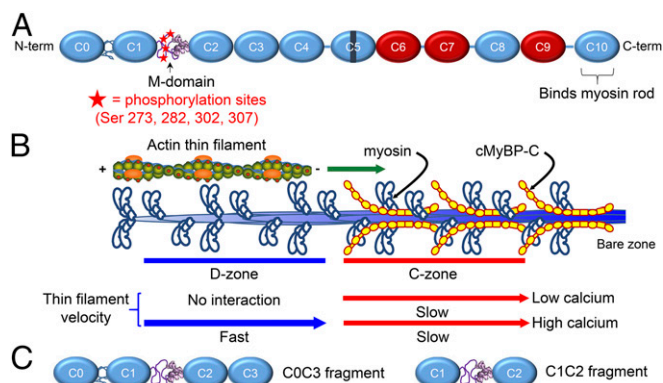


Fig. 1. Structure and function of cMyBP-C. (A) Schematic diagram of full-length cMyBP-C. Domains C1 and C2 are connected by the M-domain, containing an intrinsically disordered N-terminal region with four phosphorylatable serines, and a more structured C-terminal half. Ig-like domains are shown in blue, and FN-like domains are shown in red. (B) Illustration of half of a native thick filament with a native thin filament landing on the tip of the thick filament and being translocated through the D- and C-zones at the different speeds indicated, as observed in the TIRFM experiments. (C) Wild-type C0C3 and C1C2 N-terminal fragments used in motility, AFM, and EM assays. Phosphomimetic counterparts to each fragment were expressed containing aspartic acid substitutions for the four serines highlighted in A.

filaments (21, 23). The actin filaments used in this assay are short 250-nm shards, which allow independent probing of thick filament areas with and without cMyBP-C (the C- and D-zones, respectively) (Fig. 1B). Using this assay, we showed that the sliding velocity of bare F-actin filaments was slowed within the C-zone of thick filaments and that the degree of slowing was reduced by phosphorylation of the four serines within the M-domain (21). Next, we examined the motion of native thin filaments containing the calcium-regulatory troponin-tropomyosin complex over native thick filaments. We found that cMyBP-C in the C-zone activated the native thin filaments at low calcium levels (Fig. 1B) and that the level of cMyBP-C-induced activation was lessened by phosphorylation (23). These findings collectively indicated that phosphorylated cMyBP-C is a less potent modulator of actomyosin interactions, as also has been demonstrated in other *in vitro* model systems (20, 22).

Here, using *in vitro* motility assays in combination with atomic force microscopy (AFM) and electron microscopy (EM), we show that phosphorylation attenuates cMyBP-C's function by multimodal structural ordering within the M-domain, which in turn affects long-range global interactions between cMyBP-C's N-terminal domains. These phosphorylation-dependent structural changes suggest a molecular basis for cMyBP-C's ability to modulate cardiac contractility. In addition, we identify another novel modulatory mechanism by which calcium limits these intra- and interdomain structural interactions and the ability of cMyBP-C phosphorylation to affect function. Our findings suggest that phosphorylation and calcium can antagonistically fine-tune cMyBP-C's modulation of cardiac contractility, so that phosphorylation plays its greatest role when calcium levels within the sarcomere are low. The mechanistic phenomena described here may apply to additional proteins of the contractile machinery that are tuned by phosphorylation and possibly to other biological systems regulated through phosphorylation and changes in intracellular calcium levels.

Results

The Effect of cMyBP-C Phosphorylation on Maximal Thin Filament Sliding Velocity Is Modulated by Calcium. We applied our TIRFM-based assay to visualize the sliding of fluorescently labeled, troponin- and tropomyosin-containing native thin filaments along myosin thick filaments with high temporal (8.3-ms) and spatial (30-nm) resolution (Fig. 1B). The maximal sliding velocity of native thin filaments at high, saturating levels of calcium (0.01 mM free Ca^{2+} , 100 μM ATP, 22 °C) was slowed by 56% within the C-zone of thick

filaments containing cMyBP-C with significant levels of endogenous phosphorylation (~64% at each site) (21) (Fig. 2A). This slowing was similar to that previously described for bare actin filaments in the C-zone of native thick filaments (21). However, in contrast to our prior studies showing the ability of dephosphorylated cMyBP-C to inhibit further the sliding velocity of bare actin filaments in the C-zone (21), the sliding velocity (at high calcium levels) of native thin filaments over thick filaments containing dephosphorylated cMyBP-C (~22% at each site) (21) was equal to that over thick filaments containing endogenously phosphorylated cMyBP-C ($P > 0.05$) (Fig. 2A). This unanticipated finding that cMyBP-C phosphorylation did not modulate maximal native thin-filament sliding velocities suggested that either the presence of the troponin and tropomyosin regulatory proteins on the thin filaments or calcium itself limits the ability of phosphorylation to modulate cMyBP-C's inhibitory function.

To test whether calcium was the cause, we used a conventional *in vitro* motility assay, with a surface of randomly oriented myosin molecules, to examine the inhibitory effect of bacterially expressed cMyBP-C N-terminal fragments (wild-type C0C3, containing domains C0–C3, and a phosphomimetic mutant, C0C3^{4D}) on the sliding velocity of native thin filaments and bare actin filaments (lacking regulatory proteins) over a range of calcium concentrations (Fig. S1 and Fig. 2B). These fragments represent the extremes of cMyBP-C phosphorylation, the C0C3 fragment being unphosphorylated and the mutant C0C3^{4D} fragment with aspartic acid substitutions for the four phosphorylatable serines in the M-domain representing the fully phosphorylated state. In addition, they have been shown to recapitulate the modulatory effects of intact cMyBP-C both in the context of a native thick filament and in the more simplified *in vitro* motility assay (21, 24), with the mutant C0C3^{4D} fragment both structurally (16) and functionally (18, 21) identical to fragments fully phosphorylated by protein kinase A.

Over the upper range of physiological calcium levels (0.5–1.2 μM free Ca^{2+} , 100 μM ATP, 22 °C), the addition of 1 μM unphosphorylated wild-type C0C3 and phosphomimetic C0C3^{4D} fragments had similar ($P > 0.05$) effects on the velocity and fraction of

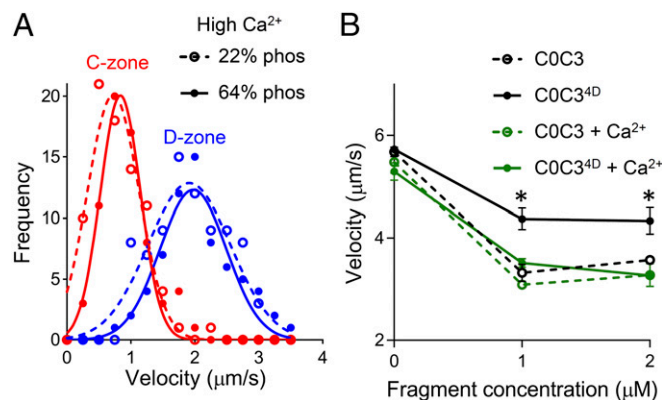


Fig. 2. Effects of cMyBP-C phosphorylation and calcium on actin-filament sliding. (A) Frequency-velocity histograms and Gaussian fits for initial native thin-filament sliding velocity in the D-zone (blue) and C-zone (red) (compare with Fig. 1B). Solid symbols and lines indicate motion on wild-type thick filaments containing highly phosphorylated (~64%) MyBP-C; open symbols and dashed lines represent motion on thick filaments containing dephosphorylated (~22%) MyBP-C. Assays were carried out at 100 μM ATP and 22 °C in the presence of 0.01 mM free calcium. (B) Sliding velocities of bare actin filaments on the surface of randomly oriented myosin molecules (skeletal muscle proteins) in the presence of unphosphorylated wild-type C0C3 (open circles, dashed lines) and phosphomimetic C0C3^{4D} (closed circles, solid lines), with (green) and without (black) 0.1 mM free calcium. The assay was carried out at 1 mM ATP and 30 °C. * $P < 0.01$ compared with C0C3, Student's *t* test. The impact of cMyBP-C phosphorylation on sliding velocity and its partial reversal by calcium is similar for actomyosin from cardiac (A and Fig. S1) and skeletal (B) muscles, suggesting that calcium acts directly on cMyBP-C.

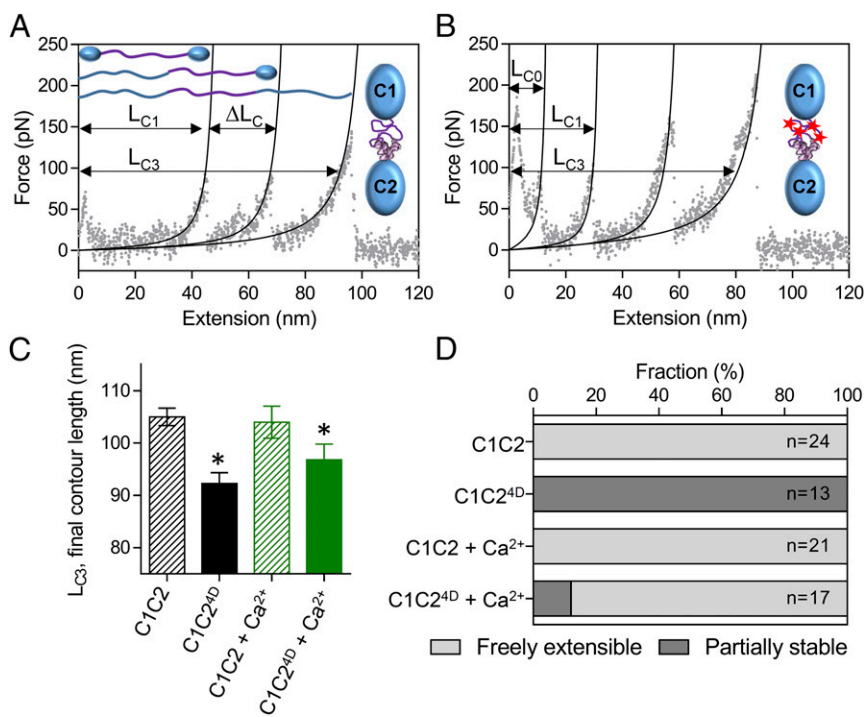


Fig. 3. Effects of phosphorylation and calcium on the structure of the M-domain. (A and B) AFM force: extension curves for single unphosphorylated wild-type C1C2 (A) and phosphomimetic C1C2^{4D} (B) molecules in the absence of calcium. The initial contour length of the freely extensible components (L_{C1}) and final contour length of the fully extended molecules (L_{C3}) are indicated, as is the change in contour length associated with the preceding unfolding event (ΔL_C). (C) The final contour lengths of the C1C2 and C1C2^{4D} fragments (L_{C3} in A and B) in the absence and presence of 0.1 mM free calcium. * $P < 0.01$, L_{C3} vs. C1C2, Student's t test. (D) The relative stability of the M-domain within the single C1C2 and C1C2^{4D} molecules in the absence and presence of 0.1 mM free calcium as determined from the number of molecules showing three (freely extensible M-domain) or four (partially stable M-domain) peaks in the force:extension curves.

native thin filaments sliding over myosin (Fig. S1), as anticipated from the thick filament-based assays containing phosphorylated cMyBP-C (Fig. 2A). To confirm further that calcium was directly affecting the function of the phosphorylated cMyBP-C (Fig. 2A) and phosphomimetic C0C3^{4D} fragments (Fig. S1), we repeated the motility assay with non-calcium-regulated F-actin filaments and myosin isolated from chicken skeletal muscle (Fig. 2B). In the absence of calcium, the addition of at least 1 μ M of the unphosphorylated wild-type C0C3 fragment inhibited actin sliding velocity (1 mM ATP, 30 °C), and this inhibition was muted in the presence of phosphomimetic C0C3^{4D} (24% vs. 40%, $P < 0.01$) (Fig. 2B). Interestingly, calcium eliminated the phosphorylation-dependent difference ($P > 0.05$) in the inhibitory capacity between the C0C3 and C0C3^{4D} fragments (Fig. 2B) so that the C0C3^{4D} was equally inhibitory. In control experiments lacking fragments, calcium had no effect ($P > 0.05$) on the sliding velocities of bare actin filament (Fig. 2B). We conclude that calcium directly limits the ability of phosphorylation to modulate cMyBP-C's inhibitory function rather than indirectly affecting the actomyosin system. Based on prior structural (10) and mechanical (16) studies showing that phosphorylation reduces the mechanical extensibility of the M-domain, we hypothesized that calcium must ablate the phosphorylation-induced structural changes, in turn affecting cMyBP-C function.

Calcium Increases the Extensibility of the Phosphorylated M-Domain.

We investigated the effect of calcium on the mechanics of the M-domain using atomic force spectroscopy. To obtain the cleanest data (with the smallest number of force-extension peaks), we examined the extensibility of bacterially expressed wild-type C1C2 (Fig. 1C) and phosphomimetic C1C2^{4D} fragments. C1C2 is the shortest fragment of cMyBP-C that maintains the native connections between the M-domain and its flanking Ig domains. With only C1 and C2 serving as attachment handles for pulling on the molecule, the mechanics of the M-domain can be isolated. C1C2 molecules (Fig. 1C) were adsorbed onto a glass coverslip; then one end of the molecule was lifted off the surface by retraction of a non-specifically attached silicon nitride atomic force microscope (AFM) probe. Force:extension traces of the wild-type C1C2 fragments showed three distinct peaks (Fig. 3A). The rising phase of the first peak (L_{C1}) resulted from the initial lengthening of the freely extensible components within the M-domain and the subsequent drop

from the unfolding of one of the Ig-like domains (16). The next peak (L_{C2}) resulted from the lengthening of the now unfolded Ig-like domain and the subsequent drop from the unfolding of the other stable Ig-like domain. The final peak (L_{C3}) was associated with full extension of the unfolded molecule before its detachment from the glass surface or probe (Fig. 3A). The length of L_{C3} (105.0 ± 8.2 nm; mean \pm SD) was consistent with the expected length (107.8 nm) of a fully linearized peptide chain (308 amino acids \times 0.35 nm per amino acid), suggesting that all secondary and tertiary structure was removed by pulling with ~ 200 pN of force applied by the atomic force microscope. In comparison, force:extension traces of the C1C2^{4D} fragments showed an additional peak (L_{C0}) and drop that presumably resulted from the unfolding of a portion of the M-domain (Fig. 3B) that was partially stabilized by phosphorylation, as described previously for longer C0C3 fragments (16). Although phosphomimetic substitution had no effect on the lengths of the unfolded Ig-like (C1 and C2) domains (ΔL_C) (Table S1), it reduced the final contour length (L_{C3}) of the fully unfolded molecule by 13 nm (Fig. 3C). This reduction was suggestive of highly stable, phosphorylation-dependent structural ordering within the M-domain that could not be extended by the force applied with the atomic force microscope.

To determine whether calcium altered the effect of phosphorylation on the development of the highly stable nascent structure and/or the partial stability of the M-domain, 0.1 mM of free calcium was added to the assay. The presence of calcium had no significant effect ($P > 0.05$) on the final contour length or extensibility of the wild-type C1C2 fragments (Fig. 3C and D). However, calcium resulted in an 88% reduction in the fraction of C1C2^{4D} fragment force:extension traces that exhibited the unique initial unfolding peak, indicating that calcium inhibits the phosphorylation-induced stabilization of the M-domain (Fig. 3D). Despite this change in stability, the final contour lengths for the C1C2^{4D} fragments were not significantly different ($P > 0.05$) in the presence or absence of calcium and still were shorter ($P < 0.05$) than the wild-type C1C2 fragments (Fig. 3C). This result suggests that phosphorylation has multiple effects on the structure of the M-domain, with calcium affecting only a subset of these changes that are detected in the measurement of partial mechanical stability (Fig. 3D). Could these changes in stability have long-range effects on cMyBP-C's structure to affect its function?

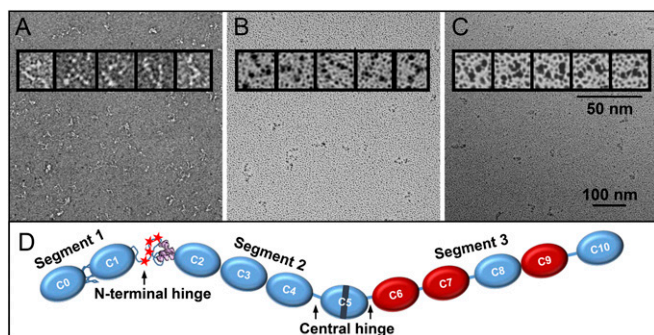


Fig. 4. Localization of a hinge point within the M-domain. (A) Negatively stained EM image of full-length baculovirus-expressed cMyBP-C. (Insets) Single molecules with visible Ig and Fn domains. (B) Rotary-shadowed EM image of full-length cMyBP-C molecules. (Insets) Examples of bent rods with one and two hinge points. (C) Rotary-shadowed EM image of full-length cMyBP-C after treatment with calpain to remove the N-terminal domains. (Insets) Molecules with only a single hinge, resulting from site-specific truncation of the N-terminal segment. (D) Schematic diagram of cMyBP-C showing the location of two hinge points connecting more rigid segments.

cMyBP-C Contains a Hinge Point Within the M-Domain. To test the hypothesis that these local phosphorylation-dependent changes in the M-domain (Fig. 3) affect the overall structure of cMyBP-C's N terminus, we visualized single cMyBP-C molecules in the absence of calcium using negative staining and rotary shadowing EM. Baculovirus-expressed full-length cMyBP-C molecules imaged by negative staining adopted multiple conformations ranging from elongated flexible molecules with hinge points, similar to chicken cMyBP-C (25), to amorphous structures (Fig. 4A). The elongated molecules comprised globular units 3–5 nm in diameter, consistent with a linear arrangement of individual ~4-nm Ig and Fn domains (26). Comparable images of Ig and Fn domains have been obtained by EM of titin (27) and fibronectin molecules (28).

Although individual Ig and Fn domains were not as readily detected by rotary shadowing EM (Fig. 4B), the images showed cMyBP-C molecules with similar conformations. These included elongated bent rods with up to two hinge points (50%, $n = 200$), shorter, more rigid straight rods that were potentially folded molecules (31%), and amorphous structures that could not be classified (19%). Of the elongated bent-rod structures, 60% contained a single central hinge creating a V-like conformation, as previously observed for skeletal myBP-C (29). The remaining 40% contained a second hinge point near one end of the molecule that created a third short segment that has not been previously reported. The segments were 8 ± 2 nm, 15 ± 3 nm, and 20 ± 2 nm in length (Table 1).

To determine whether this second hinge point was within the M-domain (a possibility suggested by its proximity to one end of the molecule), we digested the full-length cMyBP-C with calpain. Calpain specifically cleaves between R266 and R270, located near the first phosphorylation site (S273) (21), releasing a 29-kDa fragment comprising the C0–C1 domains and the first 17 amino acids of the M-domain (this fragment has also been referred to as a “40-kDa fragment” based on its migration on SDS/PAGE) (Fig. S2). After calpain cleavage (Fig. S2), both elongated bent rods (35 ± 3 nm, $n = 100$) and short rod-like molecules (9 ± 2 nm, $n = 20$) were observed in rotary-shadowed EM images (Fig. 4C). The majority (94%) of the elongated molecules contained a single hinge point, creating two segments. The segments on either side of the hinge were asymmetric and when grouped accordingly had lengths of 15 ± 2 nm and 20 ± 2 nm, similar to the lengths of the longer segments observed in intact cMyBP-C (Table 1). The shorter (9-nm) rods were similar in length to the 8-nm segment in intact cMyBP-C (segment 1 in Table 1), suggesting that the hinge point creating this segment is located near the calpain cleavage site within the M-domain (Fig. 4C). With one hinge point located within the M-domain, we can use the lengths of each segment (Table 1) and the ~4-nm size of the Ig and Fn domains (Fig. 4A) to predict that the central hinge point

occurs near the C5 domain (Fig. 4D). Based on our current resolution (± 2 nm), we cannot conclusively determine whether this hinge occurs between the C4 and C5 domains, within the disordered ~30-aa cardiac-specific insert in C5 (30), or between the C5 and C6 domains as previously predicted (31). This central hinge point may be involved with the extension of the molecule from the thick filament, and the N-terminal hinge may function to alter how the N-terminal domains interact with actin and/or the myosin S2 region.

cMyBP-C Phosphorylation Promotes Bending of the N Terminus at the M-Domain. With the presence of a hinge point in the M-domain, it is possible that the phosphorylation-induced partial stability that is observed previously with C0C3 (16) and presently with C1C2 in AFM might promote changes in the structural orientation of the N-terminal domains. Therefore, we examined the structure of bacterially expressed wild-type C0C3, PKA-treated C0C3, and phosphomimetic C0C3^{4D} fragments (Fig. 1C) by rotary shadowing EM (Fig. S3A and Fig. 5A and B). For each fragment, three distinct classes of molecules were observed: extended rods, bent rods with a single hinge point, and compact/closed structures (Fig. 5C). To quantify the relative distribution of these structures in the wild-type and phosphomimetic fragments, we measured the distances between the ends of the molecules in each image (Fig. 5A–C and Table 2). The majority (85%) of the wild-type C0C3 molecules showed the extended (>14 nm between the two ends) or bent (7–14 nm between ends) conformations (Table 2). The bent rods contained segments of 8 ± 1 nm and 11 ± 1 nm, the 8-nm segment being similar to that observed for segment 1 in intact cMyBP-C (Fig. 4D and Table 1). In contrast, the majority (61%) of the C0C3^{4D} molecules exhibited the compact structure rather than the extended or bent conformations (Fig. 5B and Table 2). The phosphorylation-induced closure of the N terminus around this hinge suggests that local changes in the structure of the M-domain (Fig. 3) promote global changes in orientation of the N-terminal domains seen in these rotary shadowed images.

Phosphorylation-Induced Closure of the N-Terminal Domains Is Attenuated by Calcium. Does calcium's ability to destabilize the phosphorylated M-domain (Fig. 3D) mitigate the closure of cMyBP-C's N-terminal domains? If so, this effect could account for the loss of phosphorylation-dependent modulation of thin filament velocities in the motility assays in the presence of calcium (Fig. 2). To address this question, rotary shadowed EM images of the wild-type C0C3, PKA-treated C0C3, and phosphomimetic C0C3^{4D} fragments were obtained in the presence of 0.1 mM free calcium (Fig. 5D and E and Fig. S3B). The addition of calcium resulted in fewer of the PKA-treated and phosphomimetic molecules adopting the compact conformation. When quantified, there was an 89% reduction in the fraction of C0C3^{4D} molecules adopting the compact conformation, with the distribution of molecules in the extended and bent rod conformations being similar to that in the wild-type C0C3 fragments regardless of the presence of calcium (Table 2). This calcium-induced reopening of the C0C3^{4D} molecules likely results from a disruption in global interactions within the N-terminal domains (Fig. 3D) and could explain how calcium ablated the ability of phosphorylation to modulate the function of cMyBP-C in our motility assays (Fig. 2).

Table 1. Lengths of rotary shadowed cMyBP-C and C0C3 segments

	Segment 1, nm	Segment 2, nm	Segment 3, nm
cMyBP-C	8 ± 2	15 ± 3	20 ± 2
cMyBP-C/calpain		15 ± 2	20 ± 2
C0C3	8 ± 1	11 ± 1	

Segment numbers refer to labels in Fig. 4D. Lengths are mean \pm SD; 50 molecules were measured in each case.

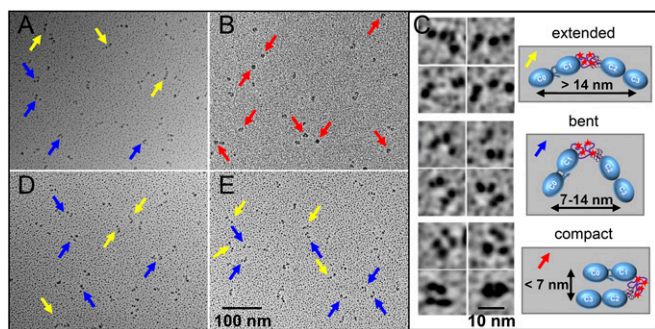


Fig. 5. Effects of phosphorylation and calcium on the orientation of the N-terminal domains. (A and B) Rotary-shadowed EM images of nonphosphorylated wild-type C0C3 (A) and phosphomimetic C0C3^{4D} (B) fragments in the absence of calcium. (C) Representative EM images (Left) and schematic diagrams (Right) of the extended rods (Top), bent rods (Middle), and compact/closed structures (Bottom) classified in each EM image. (D and E) Rotary-shadowed EM images as in A and B for C0C3 (D) and C0C3^{4D} (E) fragments in the presence of calcium. Yellow, blue, and red arrows point to molecules with extended, bent, and compact structures, respectively.

Discussion

Phosphorylation sites are commonly found in intrinsically disordered regions of proteins, with phosphorylation having both local effects on the order of secondary structures and global effects on the orientation of tertiary states (32–34). Such structural rearrangements can result in changes in the affinity for binding partners (33, 35) and/or the mechanics of the phosphoprotein in the bound state (32, 36). Each of these mechanisms allows exquisite tuning of the protein interactions involved in complex biological processes.

Here, we determined that phosphorylation of four residues within an intrinsically disordered region of cMyBP-C's M-domain significantly impacts cMyBP-C's structure, in turn tuning cMyBP-C's ability to modulate actomyosin motility. Phosphorylation-dependent structural changes within the M-domain were evident by a reduction in the C1C2 fragment's final contour length (Fig. 3C) and a change in mechanical extensibility (Fig. 3D) and were manifest beyond the M-domain by shifts in the conformation of cMyBP-C's N terminus (Fig. 5A and B). The decrease in contour length upon phosphorylation suggests the formation of secondary and/or tertiary structures within the M-domain that are so stable that they cannot be unraveled by the AFM pulling forces (~200 pN). The formation of these nascent structures may partially stabilize the M-domain by their surface charge distribution, allowing more electrostatic interactions with either the three-helix bundle or other neighboring domains. In support of this notion, previous AFM studies showed that the phosphorylation-dependent mechanical stability of the M-domain was sensitive to ionic strength (16). Importantly, these local alterations within the M-domain appear to have long-range, global effects on the equilibrium between the extended and compact conformational states that cMyBP-C's N terminus adopts (Fig. 5A and B). Specifically, phosphorylation shifts the N-terminal domains (C0C3) from the extended conformation to a compact conformation as the C0C3 bends in half at a hinge point near the sites of phosphorylation (Fig. 6). Such conformational closure may directly control the accessibility of binding sites for both actin and/or myosin S2, allowing phosphorylation to limit cMyBP-C's affinity for either partner, as previously described (17–19), and/or directly alter the mechanics of cMyBP-C in the structurally bound state (32, 36). Either scenario could explain the phosphorylation-dependent reduction in cMyBP-C's inhibition of actomyosin motility (Fig. 2).

Interestingly, the presence of calcium at the concentrations found at the peak of cardiac muscle contraction can effectively reverse the impact of phosphorylation on the equilibrium of the N-terminal conformational state (Figs. 5E and 6), the partial stability of the M-domain (Fig. 3D), and the modulation of actomyosin motility (Fig. 2 and Fig. S1). Calcium ions are positively charged and thus may simply shield negative charges within the phosphorylation-dependent nascent structure that partially stabilizes the M-domain

Table 2. Phosphorylation and calcium affect C0C3 structure

	Extended, %	Bent, %	Compact, %
C0C3	39	46	15
C0C3 ^{4D}	5	34	61
C0C3 + Ca ²⁺	42	49	9
C0C3 ^{4D} + Ca ²⁺	37	46	17

One hundred molecules were measured in each case.

(Fig. 3D). Alternatively, phosphorylation may result in the formation of a calcium-binding motif within the M-domain. By either mechanism, the presence of calcium could shift the C0C3^{4D} fragment's conformational equilibrium back to the extended, rod-like conformation (Fig. 5E and Table 2) favored by C0C3, thus explaining why the C0C3^{4D} and C0C3 fragments become functionally indistinguishable in the motility assay when calcium is present.

The complex intersection of posttranslational modification and calcium signaling that we report has broad implications for our understanding of cMyBP-C's role in modulating cardiac contractility. The interplay between phosphorylation and calcium on cMyBP-C structure and function must be dynamic and allow fine tuning of cMyBP-C's mechanics within every heartbeat, because calcium ebbs and flows within each cardiac muscle cell. Although cMyBP-C's *in vivo* binding partner(s) have yet to be defined, cMyBP-C may serve at least two distinct functional roles: to enhance the activation state of thin filaments at low calcium concentrations and to limit the maximal activity of actomyosin interactions once the sarcomere is fully saturated with calcium (23). We recently have proposed a model based on these roles in which the localization of cMyBP-C to the sarcomeric C-zone serves to sensitize the thin filaments to calcium, helping correct an intrinsic inhomogeneity in calcium activation during the onset of contraction (23). Then, at the peak of contraction, when calcium levels have increased, cMyBP-C serves a second role as a governor that effectively throttles back actomyosin activity. Without the effects of calcium on cMyBP-C's structure, phosphorylation of cMyBP-C at normal *in situ* levels would partially mitigate both these modulatory roles (21, 23). However, because of the antagonistic interplay between phosphorylation and calcium, physiologically saturating levels of calcium would, in turn, tend to reverse the impact of phosphorylation on cMyBP-C structure and function so that cMyBP-C remains a potent modulator of contractility, as demonstrated *in vitro* (Fig. S2). Thus, phosphorylation and calcium together fine-tune cMyBP-C's modulatory function through changes in cMyBP-C's structure.

Multiple cardiac proteins, including the myosin regulatory light chain, troponin I, and phospholamban, appear to be regulated by phosphorylation-dependent structural transitions similar to those observed for cMyBP-C (32). It currently is unknown whether the interplay between phosphorylation and calcium is specific to cMyBP-C or is a general phenomenon that would impact the structure and function of these other cardiac contractile proteins by a similar mechanism. In fact, it is possible that such mechanisms may occur in

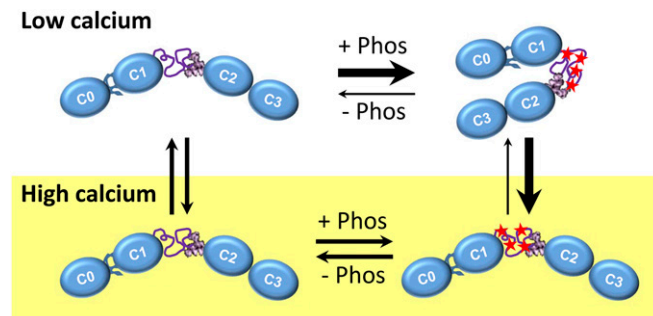


Fig. 6. Diagrams of the effects of phosphorylation on the overall structure of cMyBP-C's N terminus at low (Top) and high (Bottom) calcium levels.

other biological systems, because phosphorylation and calcium are ubiquitous signaling mechanisms critical to protein function.

Materials and Methods

Detailed methods, given in *SI Materials and Methods*, are summarized here.

Proteins. Native thick and thin filaments and depolymerized myosin were isolated from FVB mouse hearts (21, 23, 37). cMyBP-C phosphorylation levels were measured by LC-MS, and, where applicable, thick filaments were dephosphorylated with λ -phosphatase (New England BioLabs) (21). Skeletal myosin and F-actin were purified from chicken pectoralis muscle (38, 39). F-actin and native thin filaments were fluorescently labeled with equimolar TRITC (37). Wild-type and phosphomimetic mutant (S273, S282, S302, and S307 to D substitution) C1–C2 (amino acids 151–448) and C0–C3 (amino acids 1–539) mouse cMyBP-C N-terminal fragments were bacterially expressed and purified (40, 41). Full-length mouse cMyBP-C cDNA was expressed in the Baculovirus-insect cell system, purified, and where applicable, truncated with calpain-1 (Calbiochem), as described in *SI Materials and Methods*.

Motility Assays. The movement of 250-nm shards of thin filaments on thick filaments was observed (100 μ M ATP, 22 °C) by TIRFM (21). The movement of thin filaments (100 μ M ATP, 22 °C) and bare actin filaments (1 mM ATP, 30 °C) on randomly oriented surfaces of mouse cardiac and skeletal muscle myosin molecules was observed by epifluorescence microscopy in the absence and presence of

wild-type C0C3 and phosphomimetic C0C3^{4D} fragments (18, 21, 42). Free calcium concentrations were determined using MaxChelator software (43).

Atomic Force Spectroscopy. The unfolding of wild-type C1C2 and phosphomimetic C1C2^{4D} fragments was observed by pulling (180 nm at 700 nm/s) with an atomic force microscope (MFP-3 Bio; Asylum Research) (16). AFM force:extension traces were identified by a fingerprinting routine (44) and then were fit using a wormlike chain model (45).

EM. Full-length and truncated cMyBP-C and wild-type, PKA-treated C0C3, and phosphomimetic C0C3^{4D} fragments were visualized by negative staining and/or rotary shadowing EM. EM grids were examined using a transmission electron microscope (FEI), and lengths were measured from digital images using the line tool in ImageJ.

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