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Modulation of striated muscle contraction by binding of myosin binding protein C to actin

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Myosin binding protein C (MyBP-C or C-protein) is a protein of the thick (myosin-containing) filaments of striated muscle thought to be involved in the modulation of cardiac contraction in response to β-adrenergic stimulation. The mechanism of this modulation is unknown, but one possibility is through transient binding of the N-terminal end of MyBP-C to the thin (actin-containing) filaments. While such binding has been demonstrated in vitro, it was not known until recently whether such a link between thick and thin filaments also occurred in vivo. Here we review a recent paper in which electron microscopy (EM) is used to directly demonstrate MyBP-C links between myosin and actin filaments in the intact sarcomere, suggesting a possible physical mechanism for modulating filament sliding. Molecular details of MyBP-C binding to actin have recently been elucidated by EM of isolated filaments: the results suggest that MyBP-C might contribute to the modulation of contraction in part by competing with tropomyosin for binding sites on actin. New results on the structure and dynamics of the MyBP-C molecule provide additional insights into the function of this enigmatic molecule.

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Myosin binding protein C (MyBP-C or C-protein), an accessory protein of the thick filaments of vertebrate striated muscle, has been the focus of intense interest ever since the discovery that mutations in the cardiac isoform are a major cause of the familial heart disease, hypertrophic cardiomyopathy (HCM).[1,2] Mutations in the slow skeletal isoform also lead to skeletal muscle myopathy: distal arthrogryposis type 1,[3,4] a disease of the distal limbs, is thought to result from restricted movement of the fetus in the uterus. MyBP-C has a beads-on-a-string structure consisting mainly of domains of the immunoglobulin (Ig) and fibronectin type III (Fn3) families (**Fig. 1**).[7] The C-terminal domains anchor MyBP-C to the myosin tails and titin in the thick filament backbone, while the N-terminal region has been shown to interact with both the initial part of the myosin tail (subfragment 2, S2),[8-10] with the myosin regulatory light chain,[11] and also with actin.[12-19] Immuno-EM studies have shown that MyBP-C is located on 7–9 stripes, 43 nm apart, in each half of the A-band,[20-22] stripes that can also be seen directly in well-preserved unlabelled muscle (**Fig. 2**).

Although MyBP-C was discovered nearly 40 y ago,[25] its function is not yet fully understood. In the heart it appears to be involved in the modulation of contraction in response to β-adrenergic stimulation[9,26,27]; in skeletal muscle its role is unclear. One way in which MyBP-C might in principle modulate cardiac contraction is through connection to the thin filaments. Interaction with both F-actin and Ca^{2+}-regulated thin filaments (containing tropomyosin and troponin) has been shown in vitro to slow F-actin motility and to modulate the state of activity of thin filaments.[16,19] Whether such interactions occur in situ, or are merely an in vitro artifact, has recently been clarified by electron tomography of sectioned skeletal muscle.[24] Here we

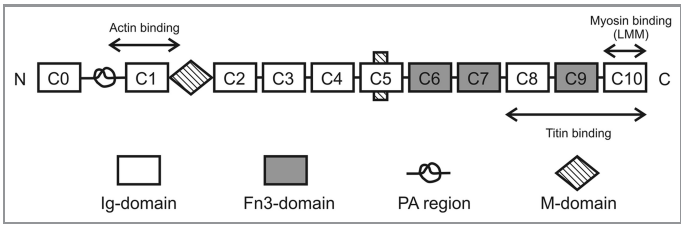

Figure 1. Domain structure of MyBP-C. The majority of this elongated protein consists of tandem Ig-like and Fn3-like, 10 kDa globular domains, ~4 nm in diameter. The cardiac isoform shown has 11 such domains together with a MyBP-C-specific "M-domain" (which includes 4 phosphorylation sites), a 28-residue insertion in the C5 domain, and a Pro-Ala-rich region (PA).[5] The skeletal isoform is similar, but lacks the N-terminal C0 domain, the C5 insertion and the phosphorylation sites. The C-terminal domains bind to the thick filament (LMM and titin),[5] while the N-terminal domains are capable of binding to actin and/or myosin S2. Mutations over the whole length of cardiac MyBP-C lead to HCM and to date 197 HCM-causing mutations have been found.[6]

review these findings—the first to directly demonstrate MyBP-C links between the two types of contractile filament. We relate these results to observations of N-terminal MyBP-C fragments bound to F-actin[28] and of the flexible and dynamic structure of the MyBP-C molecule itself.[29,30]

Three-Dimensional Organization of MyBP-C in the Sarcomere

While the periodic distribution of MyBP-C within the two central regions (C-zones) of each half thick filament has long been known, its organization in three dimensions has remained a mystery. The narrowness of the stripes suggests that the elongated MyBP-C molecule (**Fig. 1**) is oriented perpendicular to the filament axis, rather than longitudinally, but whether it wraps around the backbone, or possibly extends out toward the thin filaments is not apparent from direct inspection of the electron micrographs (**Fig. 2**). Knowing the answer to this question could provide key insights into MyBP-C function, in particular whether it interacts with thin filaments in situ. Deciphering the organization of this narrow, elongated, and labile protein in intact muscle has recently been achieved by 3D electron microscopy of exceptionally well preserved specimens.[24] Tissue structure is conventionally preserved for EM by chemical fixation using glutaraldehyde, a bi-functional protein crosslinker. Major cellular components and organelles are well preserved in this way, but fine molecular details are usually lost. It has been found experimentally that the best preservation is achieved by cryo-fixation, which can instantaneously capture 3D cell and molecular architecture by the physical process of freezing. Freezing is achieved by "slamming" tissue against a polished copper block cooled with liquid helium, which vitrifies specimens (freezes them without ice crystal damage) within a millisecond to depths of up to 20 μm. Following vitrification, specimens can be sectioned at low temperature (cryo-sectioned) then imaged in the frozen state by cryo-EM.[31] While this procedure preserves native structure better than any other, it is complex and technically challenging. An alternative method that yields excellent results is freeze-substitution: instead of cryo-sectioning, the vitrified tissue is chemically fixed at low temperature (e.g., with osmium tetroxide in acetone) as the ice gradually dissolves. This is followed by embedding in epoxy resin and conventional thin sectioning (e.g., Padron et al.[32]). This is the procedure we used to preserve thick filament 3D molecular structure in sections of frog skeletal (sartorius) muscle.[33] Comparison of the averaged Fourier transform of the A-bands from several electron micrographs showed excellent agreement with X-ray diffraction patterns of living muscle, demonstrating that we had indeed achieved high quality filament preservation (**Fig. 2**).[24]

Visualization of fine structure in normal EM sections is hampered by superposition of components at different levels in the section. This problem can be overcome by collecting images at different tilt angles in the microscope and recombining them computationally by the procedure of back projection (electron tomography).[34] Tilt series were collected from the sections of

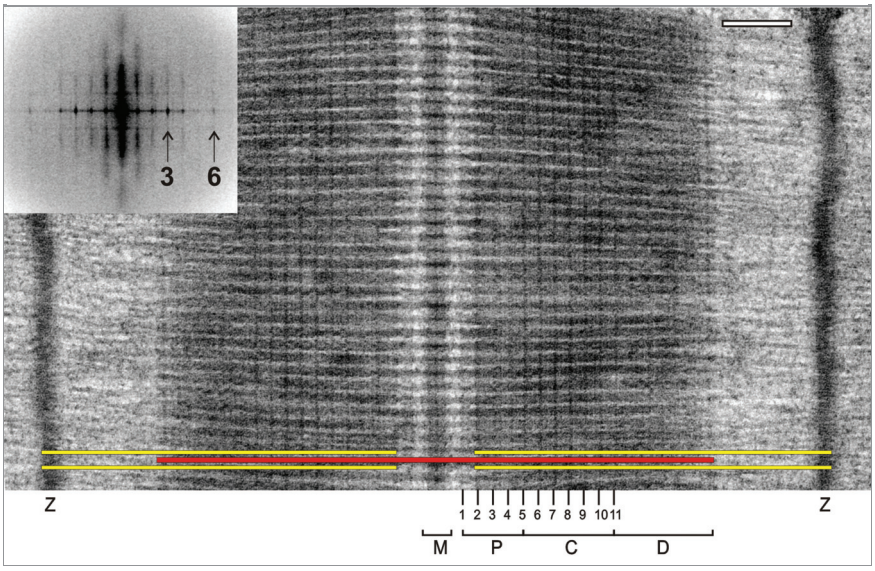

Figure 2. Longitudinal section of rapidly frozen, freeze-substituted frog sartorius muscle. Thick filaments and thin filaments run horizontally; examples are shown in red and yellow, respectively. Stripes 5–11 in the C-zone represent MyBP-C. Finer stripes between the MyBP-C stripes are myosin heads. Components of the sarcomere are labeled: M, M-band; P, P-zone; C, C-zone; D, D-zone; Z, Z-disc.[23] The inset is the averaged Fourier transform of 23 half A-bands, showing six orders of layer lines and meridional reflections coming from the thick filaments, demonstrating excellent preservation of three-dimensional order of myosin heads and MyBP-C. Inset from Luther et al.[24] Scale bar = 200 nm

rapidly frozen, freeze-substituted sartorius muscle. The high density of the MyBP-C stripes in untilted sections (**Fig. 2**) suggests that a large part of the protein is confined to a thin disc at the stripe location. However, direct inspection of the tomograms at this location does not reveal MyBP-C structure due to noise in the tomograms. To overcome this problem, we computationally extracted thick filaments from the 3D volume of three tomograms (207 filaments in all), and performed multiple rounds of alignment and averaging to obtain a final thick filament average.

Inspection of the averaged tomogram in 3D (using UCSF Chimera[35]) showed clear "crowns" of myosin heads with a 14.3 nm spacing (**Fig. 3**), long known to be present based on X-ray diffraction patterns of muscle, but never before directly visualized in muscle sections. A radially extensive envelope of density (MyBP-C) was seen at every third level of heads in the C-zone, corresponding to the location of the dense stripes in the sections (**Fig. 2**). While MyBP-C appears prominent when visualized by this surface rendering (**Fig. 3A**), depiction as a density map reveals that it is in fact weak and diffusely localized. This becomes especially evident in viewing the reconstruction from different angles (see Movie S3 in Luther et al.[24]), which shows that the intense stripes appear only when viewed exactly edge-on. Analysis of the tomogram shows that the bulk of MyBP-C density is at high radius, further from the filament backbone than the myosin heads. The density emanates at a fixed point on the filament circumference, has an initial radial component and then veers to a more circumferential direction, apparently making contact with the actin filaments (white in **Figure 3B and C**).

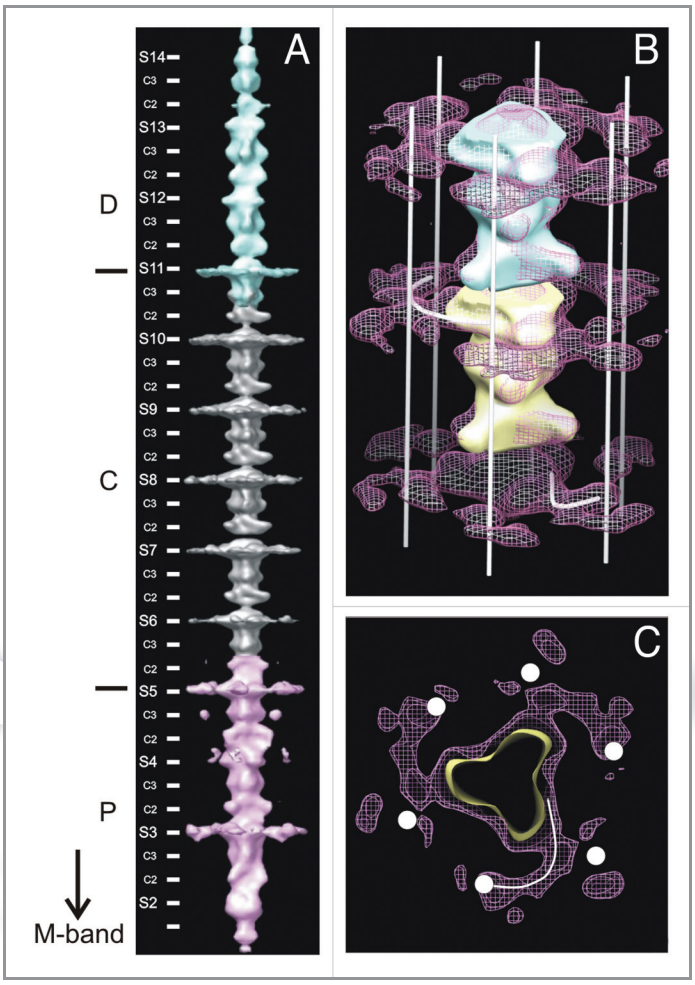

Figure 3. Three-dimensional reconstruction of averaged thick filament from electron tomography of sectioned muscle. (A) Surface view, with M-line at bottom. Myosin heads show as helically arranged projections along length of filament; MyBP-C forms "shelves" of density (S5-S11) at every third level (crown) of myosin heads, marked by c2 and c3 (c1 coincides with the MyBP-C stripes). (B) Levels 7–9 (bottom to top) of MyBP-C (pink mesh) with associated myosin heads. Straight white lines show positions of thin filaments, and curved white lines show two possible paths followed by MyBP-C as it emerges from the thick filament and runs toward its contact with the thin filaments. (C) Transverse view of (B) at level 8, showing MyBP-C as pink mesh and thin filaments as white discs. Curved white line shows possible course of MyBP-C from the surface of the thick filament to the thin filament. Figure adapted from Luther el al.[24]

These observations demonstrate directly that MyBP-C can indeed interact with thin filaments in the intact sarcomere. We conclude that previous in vitro studies of MyBP-C-thin filament interaction, and the functional conclusions derived from them, may be relevant in vivo. While the binding of MyBP-C to actin in vivo must be weak, in order to allow for filament sliding during contraction, it could have profound physiological consequences, e.g., by sensing and modulating filament sliding. Weakening of actin binding by phosphorylation of the M-domain[17] could contribute to the enhancement of cardiac contraction that occurs in response to β-adrenergic stimulation. Binding to actin could help to account for a long-standing puzzle—how MyBP-C, with its restricted location in the thick filament, can affect thick-thin filament sliding as a whole. This would not appear to occur via interaction with the small number of myosin molecules that are in contact with MyBP-C; direct connection to actin could explain effects at the whole filament level straightforwardly.

While the tomogram clearly reveals the extension of MyBP-C between thick and thin filaments, it does not provide molecular detail on the binding to the surface of either. Suggestive information on the binding of the C-terminal end to the thick filament has come from a 3D reconstruction of isolated filaments, which shows three 4-nm globular domains (coincident with the MyBP-C stripes) running longitudinally along the filament in contact with titin.[36] These may represent domains C8-C10, known to bind to the thick filament backbone; the rest of the molecule is not visualized, presumably due

to its disordering in isolated filaments. Fitting of our tomogram to the isolated filament reconstruction suggests a simple model for MyBP-C organization, in which the C-terminal three or four domains bind along the thick filament while the N-terminal half extends out and binds to the thin filaments. This arrangement argues against the organization of MyBP-C molecules into a collar around the thick filament backbone, proposed on the basis of interactions observed between C5 and C8 and between C7 and C10 in yeast 2 hybrid screens[37]; indeed there is no visible evidence in the thick filament reconstruction for circumferentially arranged 4-nm domains, even though the resolution is clearly good enough to see such detail.[36]

Mode of Interaction of MyBP-C with Thin Filaments

Structural information on the binding of MyBP-C to thin filaments has recently come from observations of actin filaments decorated with expressed N-terminal fragments. Based on neutron scattering data from filaments decorated with C0-C1-m-C2 (C0C2) fragments, it has been suggested that binding occurs by interaction of the C0 and C1 domains with subdomain 1 and the DNase I binding loop of actin.[38] However, this work was performed at relatively low ionic strength, and depended on model building of relatively low resolution data. Binding has now been studied by more direct means, using EM imaging of negatively stained F-actin decorated with the same fragment and under closer to physiological ionic strength and pH.[18] The larger filament diameter, together with alterations in the Fourier transform of decorated filaments, clearly demonstrated regular binding of the fragment; however, its location and mode of binding on actin were not revealed as 3D reconstruction was not performed. Reconstructions of filaments decorated with a number of N-terminal fragments (C0C1 with part of the M-domain, C0C2 and C0C3) at a variety of ionic strengths have now provided this missing information.[28] All fragments showed MyBP-C density over a broad portion of subdomain 1 of actin, centered over the N-terminus, with the longer fragments extending tangentially from the actin surface in the direction of the pointed end. Molecular fitting with an Ig domain atomic structure suggests that C0 and C1 bind to subdomain 1, while the M domain may bridge over subdomain 2 and possibly connect to subdomain 1 of the adjacent actin, while the C2 and C3 domains appear to lie above the actin surface (**Fig. 4A**). While this appearance agrees broadly with the neutron scattering model, the M-domain in the reconstruction appears to be attached to the actin filament, in agreement with solution data suggesting that binding to actin occurs primarily through the C1 and M-domains.[17] It is possible that the higher pH of the neutron scattering experiments caused the M-domain to dissociate. New in vitro motility and optical trap experiments localize the M-domain binding site to a cluster of highly conserved arginines within the first 17 amino acids of the domain.[19] These appear to be involved in stereospecific binding of cMyBP-C to actin, possibly by binding to actin's negatively charged N-terminus.

The most interesting finding to emerge from the reconstruction is the apparent steric clash between the C0 and C1 domains and tropomyosin. This appears to occur when tropomyosin is in the low Ca^{2+} (**Fig. 4C**) but not the high Ca^{2+} (**Fig. 4B**) position, consistent with the conclusions from neutron scattering[38] and from reconstructions of F-actin decorated with C0 and C1.[40] In vitro motility assays show that thin filaments are slowed by the presence of the C0C2 or C1C2 fragment under high Ca^{2+} conditions, whereas at low Ca^{2+} their velocity is increased.[16] The reconstruction offers a possible explanation. The absence of steric clash at high Ca^{2+} could allow uninhibited binding of cMyBP-C to actin, exerting a significant drag on thin filament sliding (consistent with a similar inhibition of the sliding of F-actin alone). In contrast, the competition of cMyBP-C and tropomyosin for part of the same region of actin at low Ca^{2+} could destabilize the blocking position of tropomyosin, thus tending to

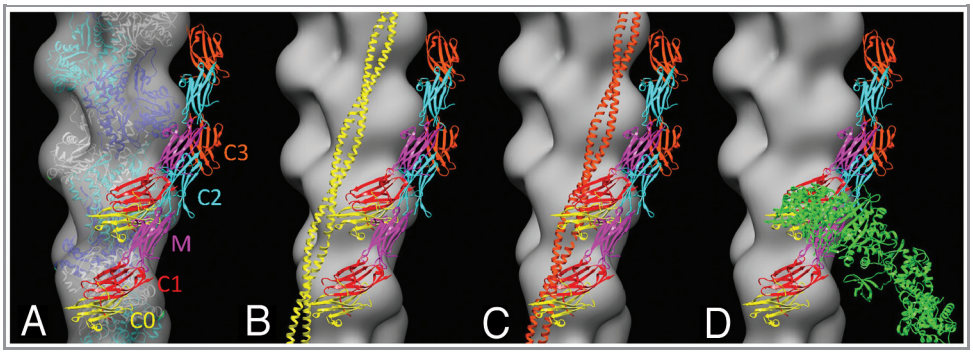

Figure 4. Binding of the N-terminal fragment C0C3 of cMyBP-C to actin. (A) F-actin reconstruction (gray) fitted with F-actin atomic model[39] (monomers colored white, blue and cyan) and showing best-fit position of two C0C3 fragments based on C0C3-decorated reconstruction. The approximate fitting of an Ig domain atomic structure to represent each of the major domains in C0C3 suggests that C0 and C1 lie on actin subdomain 1, while the M-domain crosses over subdomain 2 to the next subdomain 1, and C2 and C3 extend above the filament toward the pointed end of actin; (B and C) Fitting of tropomyosin (yellow and orange coiled coils) in high (B) and low (C) Ca^{2+} positions, showing potential steric clash of MyBP-C with tropomyosin at low but not high Ca^{2+}. (D) Fitting of myosin head (S1, green) in rigor position showing clear steric clash of S1 with C0C3. Figure adapted from Mun et al.,[28] with permission from Elsevier.

activate the thin filament, enhancing its motility. If comparable effects occur in vivo, cMyBP-C might thus contribute (together with troponin) to the balance between the low and high Ca^{2+} positions of tropomyosin.[28] It will be of interest to test this model by directly determining the effect of MyBP-C binding on tropomyosin position in regulated thin filaments.

The reconstruction also shows a major clash between myosin head and MyBP-C binding (**Fig. 4D**). Because of the low stoichiometry of MyBP-C in muscle this would be unlikely to have a major physiological effect. Phosphorylation of cMyBP-C has been shown to weaken its binding to actin, which may reduce any effect of MyBP-C on thin filament activity, and allow an increased rate of filament sliding in muscle. This could be one means by which β-adrenergic stimulation of the heart (which leads to phosphorylation of cMyBP-C) increases cardiac contractility.

Overall, these recent and earlier data suggest a model in which cMyBP-C's ability to bind reversibly to actin provides an internal load to myosin power generation in muscle, a load that can be modulated by phosphorylation of the M-domain. cMyBP-C's potential interference with tropomyosin position on actin suggests an additional means of regulating contraction.

Binding of S2 to N-Terminal Domains of MyBP-C

In addition to its actin binding capability, the N-terminal region of cMyBP-C has also been shown to interact with myosin subfragment 2 (the initial part of the myosin tail, emerging from the junction of the heads, which is thought to lie relatively loosely associated with the thick filament backbone) primarily through the M-domain.[8-10] This interaction may also play a critical role in actin-myosin interaction in intact muscle, regulated by phosphorylation of the M-domain,[9] although it would appear that this effect must be qualitatively different from that involving actin binding. The positioning of MyBP-C at only every third level of myosin heads in the middle third of each half thick filament (the C-zone, **Fig. 2**) means that only about 20% of all heads in the filament are likely to be directly affected by MyBP-C interaction. In contrast, binding of MyBP-C to actin, even in a limited region, could have a global impact, affecting sliding of the entire filament. In addition, if MyBP-C affects tropomyosin position on actin, this could be transmitted cooperatively along a substantial length of the filament, due to the significant stiffness of the tropomyosin polymer.[41,42]

While the work discussed above implicated the M-domain in binding to both actin and S2, a recent NMR study suggests that sites on the C1 domain might also serve such a dual purpose.[43] Apparent overlap between putative N-terminal actin and S2 binding sites suggests the possibility that MyBP-C might switch between binding partners in a defined way, possibly controlled by phosphorylation of the M-domain.[14,43]

Flexibility and Disorder in MyBP-C

Fascinating new insights into the structure of the isolated MyBP-C molecule, which may help to explain some of its enigmatic behavior, have emerged from two recent biophysical studies. Atomic force microscopy experiments, in which the mechanical properties of individual MyBP-C domains can be measured, show that the Ig and Fn3 domains that comprise most of the molecule are stably folded, but capable of unfolding under imposed stress, with different domains having different unfolding thresholds.[30] The M-domain stands out in being much weaker than the Ig and Fn3 domains. It appears to behave like a highly extensible spring, a property that probably relates to its predicted intrinsic disorder.[30] What function does this plasticity of MyBP-C serve? One speculation is that mechanical load on MyBP-C may affect its binding to or activation of signaling molecules. Similarly, binding sites for actin or S2 within the extensible M-domain may be modulated by stretch, becoming more exposed when MyBP-C is under tension, and these interactions may be further modulated by phosphorylation of this domain.[30] The unusual mechanical and structural properties of the M-domain suggest that it may function as an entropic spring (similar to the PEVK domain of titin), readily extending with low applied force and relaxing back to a globular structure when tension is removed. It is speculated, for example, that such behavior could contribute to the relaxation properties of the heart.[30]

Small angle X-ray scattering (SAXS) and NMR spectroscopy have provided further insights into the structural properties of cMyBP-C in solution.[29] The Pro-Ala rich region between C0 and C1 (**Fig. 1**) is found to be highly extended and flexible, consistent with the intrinsic disorder predicted for this region of the molecule (as well as the M-domain).[30] This may allow C0 to adopt multiple positions at the end of the molecule, and potentially allow even the small C0C1 segment to extend sufficiently to span between thick and thin filament surfaces.[29] By comparison with titin, which has a similar combination of defined modules and flexible/elastic proline-rich domains, it is suggested that this design of cMyBP-C may enable it to adjust to the highly dynamic environment of the contracting cardiac sarcomere.[29] Inclusion of both flexible and defined modules in the N-terminus bound to actin could clearly influence the overall transverse mechanical properties of the cardiac sarcomere.[29,44] Comparison of proline/alanine content in the Pro/Ala-rich region of cMyBP-C from different species shows a direct correlation with mammalian body size and inverse variation with heart rate.[45] These differences may be important in matching contractile speed to cardiac function in different species by differentially affecting crossbridge kinetics.[45] Alternatively, differences in the extensibility of the Pro/Ala-rich region may have evolved to generate different distributions of extended and compact states, depending on the mechanical requirements of different hearts, possibly affecting the rate of the putative switching of cMyBP-C's N-terminus between binding sites on myosin[9,11] and actin.[29]

Acknowledgments

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