This Review is part of a thematic series on **Myocyte Intra- and Extrasarcomeric Structural Proteins,** which includes the following articles:
The Giant Protein Titin: A Major Player in Myocardial Mechanics, Signaling, and Disease
The Dystrophin Glycoprotein Complex: Signaling Strength and Integrity for the Sarcolemma
Cardiac Myosin Binding Protein C: Its Role in Physiology and Disease

David Kass, Editor

Cardiac Myosin Binding Protein C
Its Role in Physiology and Disease

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Abstract—Myosin binding protein-C (MyBP-C) is a thick filament–associated protein localized to the crossbridge-containing C zones of striated muscle sarcomeres. The cardiac isoform is composed of eight immunoglobulin I–like domains and three fibronectin 3–like domains and is known to be a physiological substrate of cAMP-dependent protein kinase. MyBP-C contributes to thick filament structure via interactions at its C-terminus with the light meromyosin section of the myosin rod and with titin. The protein also has a role in the regulation of contraction, due to the binding of its N-terminus to the subfragment-2 portion of myosin, which reduces actomyosin ATPase activity; phosphorylation abolishes this interaction, resulting in release of the "brake" on crossbridge cycling. Several structural models of the interaction of MyBP-C with myosin have been proposed, although its precise arrangement on the thick filament remains to be elucidated. Mutations in the gene encoding cardiac MyBP-C are a common cause of hypertrophic cardiomyopathy, and this has led to increased interest in the protein's function. Investigation of disease-causing mutations in domains with unknown function has led to further insights into the mechanism of cMyBP-C action. This Review aims to collate the published data on those aspects of MyBP-C that are well characterized and to consider new and emerging data that further define its structural and regulatory roles and its arrangement in the sarcomere. We also speculate on the mechanisms by which hypertrophic cardiomyopathy–causing truncation and missense mutations affect the normal functioning of the sarcomere. **(*Circ Res*. 2004;94:1279-1289.)**

Key Words: cardiac myosin binding protein-C ■ hypertrophic cardiomyopathy ■ sarcomere ■ contractility

The contraction and relaxation of cardiac muscle is mediated by the sliding of interdigitating thick filaments and thin filaments. In addition to the principal thick and thin filament components, myosin and actin, the sarcomere also contains several accessory proteins that are involved in assembly, maintenance of structural integrity, and regulation of contractile activity. Mutations in the genes encoding many of these sarcomeric proteins can cause either hypertrophic cardiomyopathy (HCM) or dilated cardiomyopathy (DCM). One of the genes most commonly affected by HCM-causing mutations is that encoding cardiac myosin binding protein-C (cMyBP-C). Compared with the other sarcomeric proteins in which mutations occur, considerably less is known about the precise structure and function of this protein. In this study, we aim to provide a comprehensive review of the available functional and physiological data and to comment on the present hypotheses of the role of cMyBP-C in sarcomeric structure and in the regulation of contractility in the heart.

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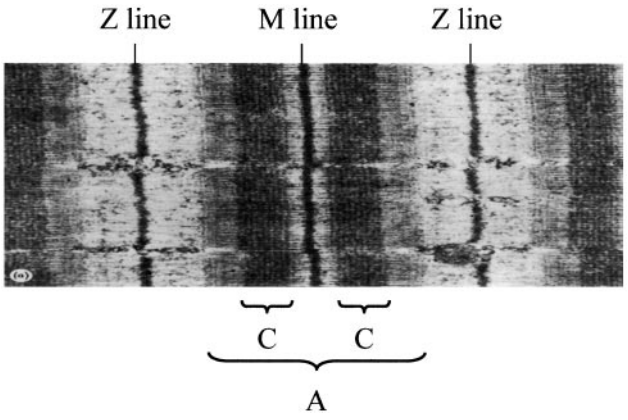

Figure 1. Rabbit psoas fibers labeled with goat antiserum to rabbit myosin binding protein C, showing the appearance of MyBP-C stripes in the C zone (C) of the A band (A) of the sarcomere. Reproduced from Craig and Offer,[3] by permission.

Characterization of MyBP-C

Initial Isolation as a Novel Myosin Binding Protein

Early x-ray diffraction and electron microscopy studies on vertebrate skeletal muscle revealed meridional reflections located solely in the A band, which were thought unlikely to originate from myosin because of their specific localization. Starr and Offer[1] consistently found a series of unidentified myosin binding proteins on separation of myosin preparations by SDS polyacrylamide gel electrophoresis. Further fractionation of these led to the identification of MyBP-C (originally termed C-protein, from impurity band C) as a myosin-associated protein,[2] a single polypeptide of molecular weight 135±15 kDa.

Localization to the A Band of the Sarcomere

The location of MyBP-C in the sarcomere was determined by antibody staining of skeletal muscle fibers.[3,4] As shown in the electron micrograph in Figure 1, 11 stripes are seen on each filament in the C zone of the A band, irrespective of filament length,[3] seven to nine of which are thought to be due to MyBP-C. The precise number of C zone stripes that contain MyBP-C (and their distribution) is dependent on muscle isoform and is not known in cardiac muscle. It was estimated that there are two to four MyBP-C molecules at each stripe, each of which are 43 nm apart (see also the studies by Rome et al[5] and Bennett et al[6]). This periodicity of MyBP-C is similar to that of myosin crossbridges (at 42.9 nm[7]), as well as that of a repeat within the A-band region of the giant protein titin (at 43 nm[8]); 43.4-nm repeats (not due to myosin heads) have also been seen in the C zone, which may correspond to MyBP-C.[9] The fact that MyBP-C can be stained with antibody indicates that at least part of it lies on the thick filament surface, whereas transverse sections of stained muscle also suggest that it wraps around the circumference of the thick filament.[3]

Early Structural Characterization of MyBP-C

Purified MyBP-C exists as a single polypeptide chain, with an extended length of 50 nm in the bovine skeletal isoform,[10] although biochemical studies indicate an ability to multimerize reversibly.[2,11] Both purified skeletal and cardiac MyBP-C molecules are predominantly V-shaped, with one arm consistently slightly longer than the other. The vertex of the V seems to have a globular shape.[11,12]

Isoforms of MyBP-C and Other Myosin Binding Proteins

Three isoforms of MyBP-C are known to exist in adult muscle: fast skeletal, slow skeletal (originally described as MyBP-X), and cardiac; separate genes encode each isoform. The genes for the human fast (*MYBPC2*) and slow skeletal (*MYBPC1*) isoforms are on chromosomes 19q13.33 and 12q23.3, respectively,[13] and the gene for human cardiac MyBP-C (*MYBPC3*) is on chromosome 11p11.2.[14] The fast and slow skeletal isoforms can be seen together in some muscle types[15,16] and can even coexist within the same sarcomere.[17] The cardiac isoform was identified during phosphorylation studies of heart muscle extract.[18]

A related protein, MyBP-H, was detected in the initial separation of myosin binding proteins.[1] It is smaller than the MyBP-Cs and, in skeletal muscle, has been localized to the third stripe of the 11 seen in the C zone.[6,19,20] As shown in Figure 2, it has high homology to the four C-terminal domains of MyBP-C, with 50% identity and a further 17% conserved amino acids.[21,22]

MyBP-C Expression Patterns

cMyBP-C is expressed at an early stage in mammalian cardiac development[23] (gestation day 8 for mice, week 11 for

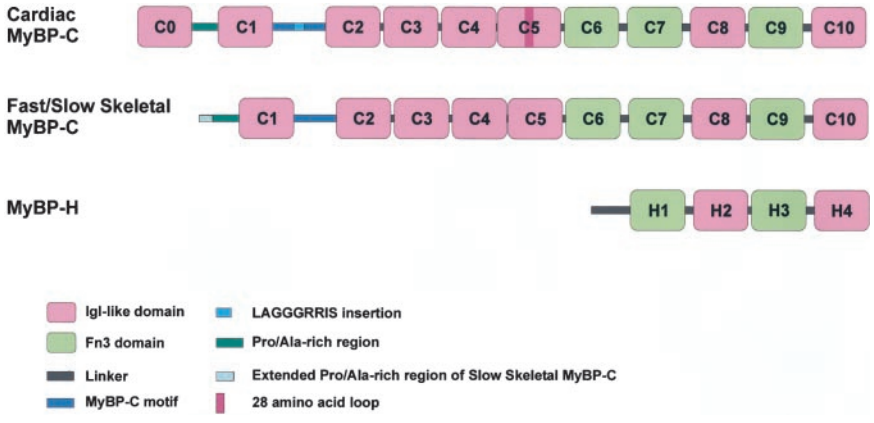

Figure 2. Schematic representation of the domain organization of myosin binding proteins. IgI-like domains are seen in pink, and Fn3 domains in green. Cardiac MyBP-C has the following isoform-specific additions: the C0 domain, the LAGGGRRIS insertion in the MyBP-C motif, and a 28-amino acid loop in the C5 domain. Fast and slow skeletal MyBP-C differ at the N-terminus, with a longer extension in the slow isoform.

the human fetus), along with myosin and titin, and at no point is either of the skeletal isoforms expressed in the heart. In skeletal muscle development, the skeletal MyBP-C isoforms are seen later, after myosin and titin expression, with slow skeletal MyBP-C expression preceding fast skeletal MyBP-C expression. An embryonic form of MyBP-C has also been reported, appearing coincident with titin and myosin.[23] In both chicken and axolotl, this early form of MyBP-C in skeletal muscle is believed to be the cardiac isoform[24–26]; however, in mice, the cardiac isoform is not transcribed at all throughout skeletal muscle development.[27]

MyBP-C Sequence Determination

The sequences of fast skeletal MyBP-C isoforms have been determined in human, chicken, and mouse,[10,13,27–29] as have the human and mouse slow skeletal MyBP-C sequences.[13,27] Figure 2 shows that the proteins consist of 10 globular domains termed C1-C10, 7 of which are immunoglobulin I–like (IgI-like) domains, with the remaining three being fibronectin 3 (Fn3) domains.[28] A conserved linker, termed the MyBP-C motif,[14] exists between domains C1 and C2. There is also a proline/alanine-rich extension N-terminal of C1. The domain organization is conserved throughout all MyBP-Cs, as is much of the sequence, particularly in the C-terminal domains.[29] When the sequence of cardiac MyBP-C was determined (in human, chicken, and mouse),[14,30,31] it was found to have a similar domain organization to the skeletal isoforms. However, importantly, it had the following additional cardiac-specific features (Figure 2): an IgI-like domain at the N-terminus (termed C0), an amino acid sequence LAGGGRRIS within the MyBP-C motif, and a 28-amino acid insertion within the C5 domain.

MyBP-C Role in Thick Filament Formation

Binding to the Light Meromyosin Region of the Myosin Rod

Early studies confirmed the expectation that MyBP-C bound to myosin by identifying an interaction with the light meromyosin (LMM) portion of the myosin rod, the region forming the backbone of the thick filament.[32] LMM binding has been shown to occur via the C-terminal domain, C10, of all three isoforms of MyBP-C.[29,33] Myosin binding of MyBP-H also occurs via its homologous C-terminal domain, H4.[33] In vitro binding studies using either recombinant C10 or proteolytic fragments have indicated saturable binding with a stoichiometry of 0.6 to 0.7:1 ratio of C10 to LMM.[29,32–34] Positively charged amino acids on C10 are believed to be involved in the interaction with LMM,[35] which was also suggested to occur between more than one interface of the C10 domain, embedded into a surface groove on the thick filament and three to four LMM molecules. This is in apparent disagreement with the stoichiometric data quoted above.

Binding to Titin

As well as binding to myosin, MyBP-C also binds to another thick filament component, titin.[10,36–38] Titin lies axially along the length of the filament,[39] most likely as three pairs of titin molecules.[40] It has been shown that cMyBP-C domains C8-C10 bind to titin, whereas C5-C8 do not, thus localizing the titin binding domain to C9 or C10.[41] The region of titin that lies in the C zone of the A band consists of a series of 11×11-domain superrepeats, also made up of IgI-like domains and Fn3 domains.[36,42] Each repeat has a periodicity of ≈43 nm,[43] and cMyBP-C binds only to this part of the titin molecule, specifically to the first domain in each repeat.[41] It is likely that the position of MyBP-C in the thick filament is dictated by the register of these titin Ig domains, because MyBP-C, like the 11-domain superrepeats, but unlike LMM, is confined to the C zone.[8] It is interesting to note that MyBP-C does not occur in stripes 1 and 2 in the C zone, despite the presence of both LMM and the titin 11 domain superrepeat; hence, it is possible that additional factors are required to direct MyBP-C to particular positions within the C zone.

The interaction between MyBP-C, myosin, and titin has therefore been thought likely to be instrumental in the ordered arrangement of the sarcomere; although the titin/MyBP-C interaction in itself is quite weak,[41] the complex binding of all three molecules at the MyBP-C C-terminus could form a very stable structure.

MyBP-C Effect on Isolated Filament Structure In Vitro

Studies carried out by Moos et al[32] found that myosin filaments can form in vitro without MyBP-C, but addition of physiological ratios of MyBP-C resulted in increased filament length, improved structure and compactness of the filament, and a clearly distinguishable central bare zone.[44,45] Thus, at least in vitro, MyBP-C does play a role in achieving proper thick filament structure.

MyBP-C Effect on Filament Structure in Transfected Cells

Coexpression of skeletal MyBP-C and myosin heavy chain in mammalian nonmuscle (COS) cells has supported the results obtained in isolated filaments. When MyBP-C is coexpressed with myosin heavy chain, myosin filaments form long, compact filaments encircling the nucleus, rather than the diffuse spindle-shaped structures formed in the absence of MyBP-C.[46,47] Expression of truncated MyBP-C suggested that the C10 domain is important for formation of these filaments, but colocalization of the two proteins required additional domains.[47] Studies in cultured skeletal myoblasts found that domains C7-C10 were necessary for successful incorporation of MyBP-C into the A band.[48,49] Of note, C8-C10, despite including both the main titin and myosin binding sites, only allowed weak incorporation. These findings seem to show that, at least in these cell systems, the four C-terminal domains of MyBP-C are involved in thick filament integrity.

Is MyBP-C Necessary for Filament Assembly?

Two mouse models have recently been generated in which cMyBP-C has been knocked out by homologous recombination, leading to deletion of the entire coding sequence[50] or the transcription initiation site.[51] These offer an opportunity to test whether cMyBP-C is necessary for cardiac muscle function. Perhaps surprisingly, homozygous-null mice are viable. Harris et al[50] found that regular sarcomere striations were seen, and features such as the Z line, A band, and M line were distinguishable, although frequently misaligned (Figure 3). Although possible upregulation of MyBP-H was not examined, the expression

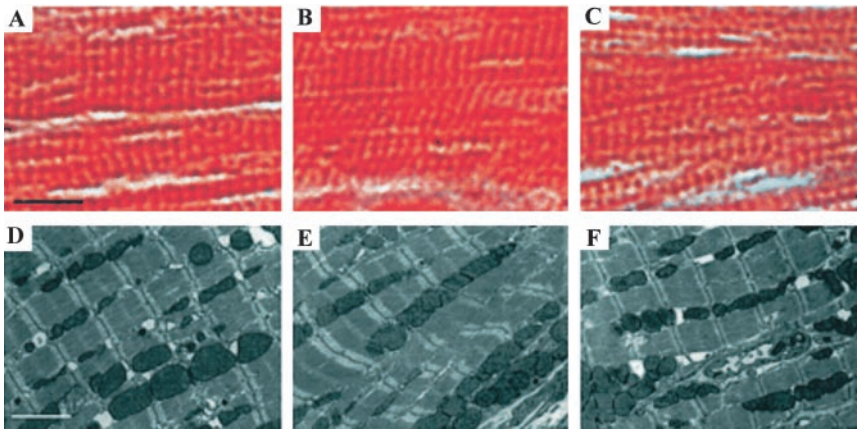

Figure 3. A through C, Light micrographs (×100 oil), stained with Masson's trichrome, of sarcomeres from wild-type (A), heterozygous (B), and homozygous (C) cMyBP-C knockout mice. Sarcomeric striations are observed in all sections, although these are less distinct in C. Bar=10 μm. D through F, Transmission electron micrographs showing that the sarcomere ultrastructure seen in wild-type animals (D) is largely preserved in heterozygous (E) and homozygous (F) cMyBP-C knockout mice. Bar=2 μm. Reproduced from Harris SP, Bartley CR, Hacker TA, McDonald KS, Douglas PS, Greaser ML, Powers PA, Moss RL. Hypertrophic cardiomyopathy in cardiac myosin binding protein-C knockout mice. *Circ Res*. 2002;90:594–601, by permission of the American Heart Association ©2002.

of skeletal MyBP-C did not increase to compensate for the lack of cardiac MyBP-C. Taken together, these in vitro, in situ, and in vivo experiments indicate that, although MyBP-C may play a role in thick filament stability, it is not absolutely essential for sarcomere formation per se. It is plausible that in the native myofilament, other factors besides MyBP-C contribute to sarcomere assembly.

Role of MyBP-C in Regulation of Contraction in the Heart

Phosphorylation of MyBP-C

Cardiac MyBP-C is one of the sarcomeric components, along with phospholamban and troponin I,[52] that can be phosphorylated in response to β-adrenergic agonists (eg, adrenaline) via cAMP-dependent protein kinase (PKA).[18,53–56] It has also been reported to be phosphorylated by an endogenous calcium/calmodulin-dependent kinase that is tightly associated with cardiac MyBP-C.[57,58] Protein kinase C has been reported to phosphorylate MyBP-C,[55,59] although not necessarily under physiological conditions.[60,61]

Gautel et al[14] have investigated the importance of phosphorylation in cardiac MyBP-C. They found that PKA could phosphorylate cardiac MyBP-C to ≈3 mol Pi per molecule and the calcium/calmodulin-dependent kinase could phosphorylate to ≈1 mol Pi per molecule.[57,58] Four potential phosphorylation sites were identified, all within the conserved MyBP-C motif between C1 and C2. These were termed sites A through D, with the following phosphorylation sequences: A, RRTS (residues 273 through 276 in the human cardiac sequence); B, RRIS (residues 282 through 285); C, KRDS (residues 301 through 304); and D, KKST (residues 360 through 363). Mutagenesis studies suggested that site D was, in fact, not phosphorylated, perhaps because of steric constraints from domain C2. Phosphorylation of site B on the cardiac-specific LAGGGRRIS insertion by PKA or calcium/calmodulin-dependent kinase[62] appeared to be requisite before sites A and C could become sterically available for phosphorylation by PKA.

Fast and slow skeletal MyBP-C are not phosphorylated to any great extent by PKA.[53,63] Because these isoforms do not have the LAGGGRRIS insertion, it is also unlikely that they are phosphorylated by the calcium/calmodulin-dependent kinase. It seems that complex, hierarchical phosphorylation may be involved in cMyBP-C–mediated regulation of contraction in cardiac muscle and that this stringent regulation does not occur in skeletal muscle.

Dephosphorylation of cMyBP-C occurs in response to cholinergic agonists, eg, acetylcholine.[56] In vitro studies in chicken have shown that this occurs predominantly via protein phosphatase 2A,[64] which is involved in the dephosphorylation of other sarcomeric proteins,[65,66] and has been shown to copurify with cMyBP-C.[57]

MyBP-C Binding to Myosin Subfragment-2 (S2)

As well as binding to LMM, a weaker interaction occurs in vitro between MyBP-C and myosin S2 (the portion of the rod joining the myosin head to the thick filament backbone).[67] This binding was shown by Gruen and Gautel[68] to be mediated by domains C1-C2, which can incorporate into the A band of the sarcomere without disrupting myofibril integrity. The binding site was localized to the MyBP-C motif and, within myosin S2, to the 126 residues closest to the junction with the myosin heads. This interaction was also common to both cardiac and skeletal MyBP-C and myosin. Importantly, the binding of cardiac MyBP-C to S2 was found to be abolished by phosphorylation of the MyBP-C motif, suggesting a possible functional role of this MyBP-C modification.[63]

Role of the C1-C2:S2 Interaction in Regulating Force Generation

When cardiac MyBP-C is phosphorylated and S2 is released, myosin crossbridges take on a position more favorable for actin binding.[69] Exogenous unphosphorylated C1-C2 added to skinned skeletal muscle fibers[70] or S2 added to permeabilized rat ventricular myocytes[71] causes the endogenous C1-C2:S2 interaction to be interrupted in a similar manner to in vivo C1-C2 phosphorylation, and there is a concomitant increase in calcium sensitivity, force of contraction, and time to half-relaxation. Similar results are also seen when MyBP-C is either partially extracted[72] or when MyBP-C is totally absent, as in the homozygous knockout mouse.[73] In studies of loaded single cardiac myocytes from this mouse model, although maximum calcium-activated force was unchanged compared with wild-type, the rate of force development increased significantly.

Winegrad and colleagues[62,74] found that a critical level of calcium was necessary for cMyBP-C to be initially phosphorylated by the calcium/calmodulin-dependent kinase at site B before phosphorylation could occur at the other sites. Because the critical calcium concentration required for monophosphorylation by this kinase was found to be less than that required for activation of contraction, it is not clear whether phosphorylation of this site is constitutive in vivo, can be tied to the normal dynamic flux of calcium in the myocyte, or requires other specific stimuli. At activating calcium concentrations, when cMyBP-C is fully phosphorylated, increased maximum force has been observed.[62]

These results suggest that cMyBP-C phosphorylation, regulated by intracellular calcium levels (to activate calcium/calmodulin-dependent kinase) and β-adrenergic stimulation (to activate PKA), determines the state of interaction of cMyBP-C with myosin S2. This affects the potential for interaction between myosin heads and actin, as hypothesized by Hofmann et al,[72] and hence is another sarcomeric regulator of force contraction. The stoichiometry of myosin and cMyBP-C in the myofibril is such that the C1-C2 region of cMyBP-C can act as a brake on only a proportion of myosin heads. However, this is sufficient to reduce overall cross-bridge cycling rates as well as to promote relaxation in the sarcomere.

A transgenic mouse has been generated in which the LAGGGRRIS insertion is deleted from cardiac MyBP-C.[75] With expressed mutant protein replacing 30% to 40% of endogenous wild-type cMyBP-C, there was a concurrent decrease in the total level of cMyBP-C phosphorylation, as would be expected. Surprisingly, there was also increased actomyosin ATPase activity and contractility, in contrast with the in vitro results discussed above. It was observed that there were increased levels of phosphorylation of wild-type cMyBP-C and also of other sarcomeric proteins, suggesting that, in this model, mechanisms may be acting to (over)compensate for the decrease in total phosphorylation of cMyBP-C. A transgenic mouse model has also been generated in which the second phosphorylation site (site B) was mutated to mimic the phosphorylated state. In better agreement with the in vitro data, isolated hearts from this model demonstrated increased cardiac output and left ventricular pressure in response to stress conditions.[76]

cMyBP-C Mutations in Hypertrophic Cardiomyopathy

Hypertrophic Cardiomyopathy

HCM was first described in detail in 1958.[77] It is an autosomal-dominant disease characterized by myocyte disarray, interstitial fibrosis, and left ventricular hypertrophy in the absence of other cardiac diseases.[78] Patients may have symptoms that include chest pain, dyspnea, and palpitation,[79] or they may be asymptomatic. There is an increased risk of sudden death, and HCM is the most common cause of sudden cardiac death in the young.[80] It is also relatively common, with up to 0.2% of the population affected.[81]

Disease-causing mutations were first mapped to the gene encoding β-myosin heavy chain (β-MHC) on chromosome 14q1.[82,83] Since this discovery, at least eight further HCM disease genes that encode components of the sarcomere have been identified either by linkage analysis or candidate gene screening (see http://www.angis.org.au/Databases/Heart/dbsearch.html). This has led to HCM being termed a disease of the sarcomere,[84] whereby the primary defect caused by the mutations is an alteration in the normal contraction or relaxation of cardiac muscle giving rise to stimuli that promote cellular hypertrophy and ventricular remodeling.

Identification of Mutations in MYBPC3 as a Cause of Hypertrophic Cardiomyopathy

Publications from two different groups in 1995 first identified the gene encoding cardiac MyBP-C (*MYBPC3*) as a HCM disease gene, the fourth such gene to be identified. Watkins et al[85] described a mutation in a splice donor site that led to the deletion of exon 30 (encoding domain C9) and a reading frame shift, followed by a premature stop codon; a 6-amino acid insertion in domain C10 was also described in another family. Bonne et al[86] identified a mutation in a splice acceptor site in intron 18, at the C4-C5 linker; the same mutation was found in two families, causing nucleotide deletions, frameshift, and a predicted truncation of the protein.

Since these first HCM-causing mutations were found in *MYBPC3*, more than 85 further mutations have been reported (see the online data supplement, available at http://circres.ahajournals.org, for a full list). Approximately two thirds of these are predicted to generate a truncated protein product caused either by mutation within a splice donor or acceptor site that leads to aberrant splicing (eg, exon skipping) or by nucleotide insertions or deletions that lead to a shift in the reading frame, giving nonsense coding sequence followed by a premature-stop codon. A few single-base mutations have been reported that result directly in a premature-stop codon. Numerous missense mutations have also been reported. These result in single amino acid substitutions throughout the cMyBP-C domains, with no particular mutational hotspots apparent. Figure 4 shows all of the missense mutations published to date as well as some of the truncation mutations.

The gene encoding cardiac MyBP-C is one of the two most commonly implicated HCM disease genes, the other being the β cardiac myosin heavy chain gene (see http://www.angis.org.au/Databases/Heart/dbsearch.html). Because different HCM disease genes are associated with somewhat differing disease profiles, the proportion of mutations in each gene varies according to the way in which patients are ascertained. A recent study[87] has suggested that the gene encoding cMyBP-C is in fact the one most commonly involved in HCM and that the prevalence of mutations in this gene has been underestimated because of its association with benign phenotypes that have not necessarily presented clinically.

Are Mutant cMyBP-C Alleles Dominant-Negatives or Null Alleles Causing Haploinsufficiency?

cMyBP-C is unique among HCM disease genes in that a large proportion of mutant alleles are predicted to encode truncated peptides; thus, whereas all other HCM genes are thought to encode poison peptides, at least some cMyBP-C mutations

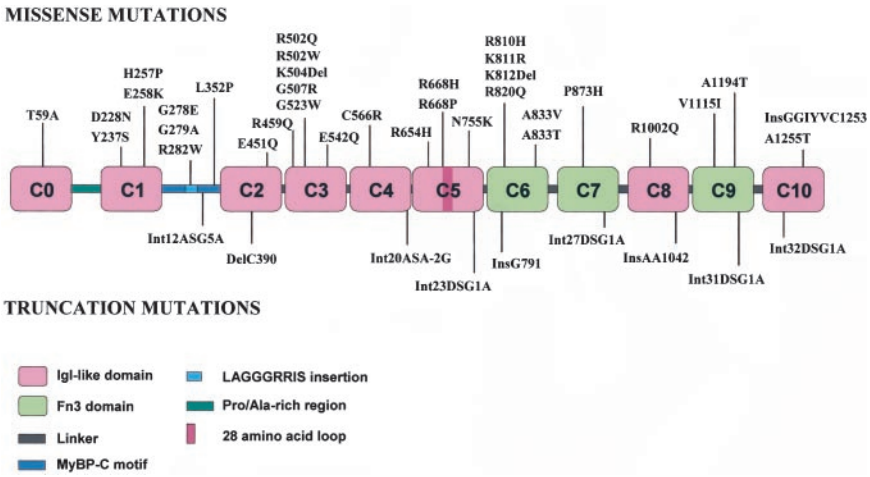

Figure 4. Published missense mutations in human cardiac MyBP-C known to cause HCM and a selection of truncation mutations. Ins indicates amino acid and nucleotide insertions; Del, nucleotide deletions. Splice site mutations are described in terms of the intron number (Int), followed by donor site (DS) or acceptor site (AS) and the nucleotide change, including its position on the consensus sequence. For a full list of published mutations and original references, see the online data supplement.

could be null alleles. Rottbauer et al[88] obtained endomyocardial biopsies from an affected patient with a splice donor-site mutation predicted to cause truncation of cMyBP-C in the C9 domain and examined mutant cMyBP-C mRNA and protein incorporation. Mutant mRNA levels were only slightly (10% to 15%) lower than wild-type levels of expression; however, no mutant protein could be detected, whereas wild-type protein seemed to be expressed at a normal level. The results suggested that the truncated protein likely was expressed (as mRNA levels were close to normal) but that the protein was then rapidly degraded and not incorporated into the sarcomere and were thus interpreted as supporting a haploinsufficiency mechanism. However, this is in contrast to several of the described mouse models (see below) in which significant quantities of incorporated truncated protein have been reported and to the effects of truncated mutants on sarcomere assembly in cell culture (see below). It remains possible that a very low proportion of mutant protein can interrupt proper function, either by incorporation/misincorporation into the thick filament or by an effect of unincorporated protein (for example by affecting the native C1-C2:S2 interaction[89]). Precedents exist for dominant-negative effects on sarcomere function from mutant proteins incorporated at $<5\%$ of total,[90] and such a low abundance of unstable truncated protein could be missed. Further, heterozygous-null mice do not show a decrease in protein expression.[50]

Most cMyBP-C missense mutations alter an amino acid that lies outside of the A-band localization region. Therefore, it is likely that these cMyBP-C mutants are incorporated into the sarcomere, although there is no direct experimental evidence from patient biopsy samples. These proteins are likely to act as "poison polypeptides" in that they are incorporated but then disrupt some aspect of normal sarcomere function. Thus, at least some (and conceivably all) cMyBP-C HCM alleles act as dominant-negative alleles.

Using HCM-Causing Mutations in cMyBP-C to Model the Disease

HCM-causing mutations have been introduced into cMyBP-C to determine how they affect function. cMyBP-C with a missense mutation (E542Q) expressed in rat fetal cardiomyocytes demonstrated the same expression and localization as endogenous cMyBP-C.[91] In contrast, but as predicted from previous studies of deletion mutants of cMyBP-C, cells expressing three different truncation alleles had diffuse localization of cMyBP-C, with approximately half of these exhibiting altered myofibrillar structure. These results are consistent with the conclusion that HCM-causing truncation mutations affect thick filament binding of cMyBP-C and suggest that this, in turn, affects sarcomere structure.

Several transgenic mice have been engineered to model the effects of HCM-causing mutations in vivo, including two transgenic mouse models that overexpress truncated cMyBP-C, either missing only the LMM binding domains or the complete A-band localization region.[92,93] cMyBP-C missing the full A-band localization region seems to be stably expressed to approximately the same level as endogenous wild-type. It does not incorporate efficiently into the sarcomere and causes increased calcium sensitivity, decreased power output, and myocyte degeneration. Conversely, cMyBP-C missing part of domain C10 was expressed at very low levels compared with the endogenous wild-type but still caused disrupted sarcomere organization and impaired fiber mechanics. A homozygous gene-targeted mouse expressing only cMyBP-C lacking the correct myosin and titin binding sites[94] showed very low levels of mutant protein incorporation (2% to 3%), but the sarcomere remained intact; the resulting phenotype seems to be that of dilated cardiomyopathy. It was suggested that, in this model, cMyBP-C may have been replaced by other proteins, such as MyBP-H. As discussed, mouse models where both alleles of cMyBP-C are knocked out are viable, although the sarcomere striations are often misaligned[50]; these homozygous-null mice also suffered from severely impaired cardiac contractility and hypertrophy. Interestingly, heterozygote mice had a similar phenotype to wild-type mice.

Structure-Function Inferences from cMyBP-C Mutations: Evidence for a C5:C8 Interaction

Missense Mutations in Domain C5

There are several domains of cMyBP-C in which HCM-causing missense mutations occur but that have no assigned function, implying that the role of cMyBP-C has further complexities. Interest in our laboratory has focused on the C5

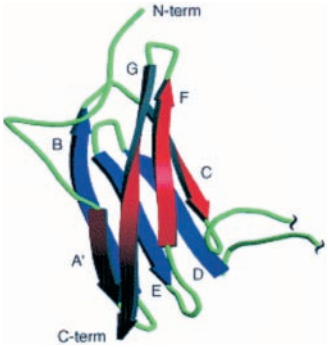

Figure 5. A secondary structure cartoon of the C5 domain of cardiac MyBP-C, reproduced from Idowu et al,[99] with permission. The 2 β-sheets are seen in red and blue, and loops and turns are seen in green. The 28-amino acid insertion is not shown, because its flexibility meant that its structure could not be determined.

domain, because it has the 28-amino acid cardiac-specific insertion, there is an apparent extended linker between domains C4 and C5, and there are presently four known disease-causing missense mutations flanking this insertion. Two of these have been studied: an arginine-to-histidine substitution at amino acid 654, with a mild phenotype,[95] and an asparagines-to-lysine substitution at amino acid 755, with a more severe phenotype and high penetrance.[96,97] The presence of the cardiac-specific insertion, as well as the proclivity for single amino acid changes in this domain to cause clinical disease, implicated an important, possibly cardiac-specific, function for this domain. It was postulated that the function of C5 would, in common with other immunoglobulin-like domains, involve protein-protein interaction.

An Interaction Between Domains C5 and C8

Yeast 2-hybrid screening of a human cardiac cDNA library showed that a C5 bait interacted with the C-terminal portion of cMyBP-C itself.[98] Deletion mapping studies identified C8 as the specific binding domain, and the C5:C8 interaction was confirmed by surface plasmon resonance. The HCM-causing mutations in C5 reduced the strength of the interaction from a K_a of $\approx 1\times10^5$ mol/L^{-1} for wild-type binding, to $\approx 6\times10^4$ mol/L^{-1} for the R654H mutant, and to $\approx 1\times10^4$ mol/L^{-1} for the N755K mutant. The extent of weakening of the C5:C8 interaction by each mutation correlates with the severity of the amino acid change in terms of charge change and with the clinical phenotype.

Structure of the C5 Domain

Idowu et al[99] have determined the structure of the C5 domain using multinuclear nuclear magnetic resonance (Figure 5). C5 has a predominant β-sheet structure, as expected for an IgI-like motif. The postulated extended linker with C4 seems not to be a linker but to make up part of the C5 structure itself at its N-terminus. The C4-C5 linker is therefore much shorter than previously expected, and the two domains are thought to be very close to each other relative to the distance between other pairs of domains. The 28-amino acid cardiac-specific insertion seems to be completely unstructured and extends away from the face of the protein. Its presence also negates some of the stabilizing interactions that usually occur between residues in an IgI domain.

When the HCM-causing missense mutations were introduced into C5, R654H seemed not to affect the structure or stability of the domain, suggesting that its disease-causing mechanism is not via protein destabilization. N755K, however, caused almost complete unfolding of C5.[99] The effects of these two mutations on the C5 structure are supportive of the binding data obtained for the interaction with C8.

Further Sarcomeric Interactions of MyBP-C

A Potential Third Myosin Binding Site on cMyBP-C

One group has suggested the possibility of a third myosin binding site within cMyBP-C.[91] It was found that expression of a human cardiac C0-C1 domain construct in fetal rat cardiomyocytes resulted in the incorporation of this fragment into the A band. Sequence analysis suggests there is a region of homology between the C0 domain (residues 59 through 103) and the LMM binding domain of myomesin (residues 72 through 116 of the human sequence), an M-band protein; the conserved amino acids in this potential binding region are also present in the mouse and chicken C0 domains. These results support the suggestion by Davis[45] of a third, low-affinity myosin binding site but at present seem difficult to reconcile with the well-documented C1-C2 interaction with myosin S2.

A Putative Interaction Between MyBP-C and Actin

Early experiments showed that skeletal MyBP-C can bind to actin filaments[100,101] and to the I band in complete sarcomeres,[102] although the interaction is much weaker than MyBP-C binding to myosin (by a factor of at least 10). More recently it has been suggested that MyBP-C may interact with actin via the proline/alanine-rich region that lies at the N-terminus of skeletal MyBP-C and between C0 and C1 of cardiac MyBP-C[103,104] (Figure 2). This region has been suggested to share homology with the N-terminal extension of essential myosin light chain and the PEVK region of titin, both of which are known to bind to actin.[105,106] In experiments in skinned trabeculae, addition of high concentrations of the C0-proline/alanine-rich region decreased maximal force. This effect was only seen when cMyBP-C was phosphorylated (ie, when C1-C2 would not be bound to myosin S2), leading Kulikovskaya et al[104] to suggest that the interaction was physiologically relevant. However, the role of the C0-C1 domains, including the proline/alanine-rich region, remains unclear, and the potential interactions of MyBP-C with actin are presently largely speculative. The possibility of the C0-C1 domains binding to other thin filament proteins or indeed thick filament proteins cannot be ruled out.

Current Models of MyBP-C Organization Within the Sarcomere

Several models hypothesizing the arrangement of MyBP-C in the sarcomere have been proposed. In his review in *Circulation Research* in 1999,[107] Winegrad proposed an arrangement

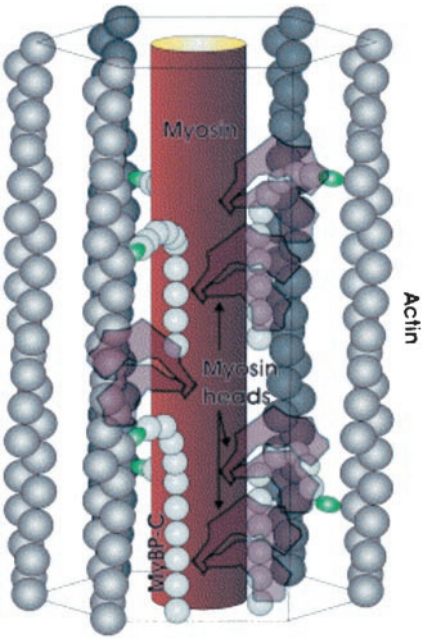

Figure 6. Model proposed by Squire et al[103] of MyBP-C arrangement on the thick filament. MyBP-C molecules (white beads representing domains) lie parallel to the myosin rod (brown). MyBP-C N-termini extend away from the thick filament toward actin (gray beads). The proline/alanine-rich region is shown in green. Myosin heads are indicated in purple. Reproduced from Squire et al, with permission.

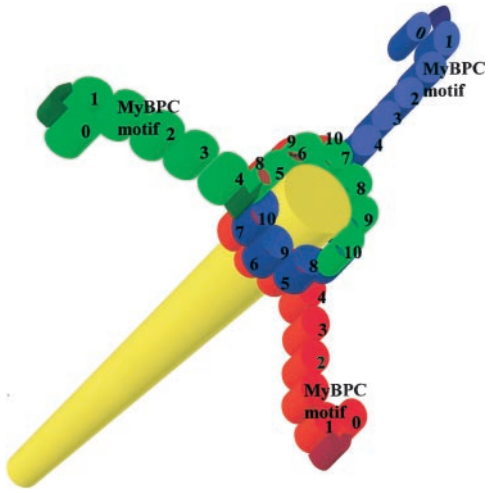

Figure 7. Proposed trimeric collar of cMyBP-C molecules around the thick filament (shown in yellow). Domains C5-C10 of each molecule overlap in a staggered parallel arrangement, stabilized by interactions between domains C5-C8 and C7-C10.

whereby three cMyBP-C molecules form a collar around the thick filament backbone, with the three C-terminal domains of one molecule binding to the three N-terminal domains of the next. Work from his laboratory has suggested that on triphosphorylation of cMyBP-C, myosin heads extend into a position more favorable for actin binding: myosin rods in the thick filament transform from being tightly packed (where the external diameter of the thick filament is 30 nm) to being loosely packed with greater order to the myosin heads (and a thick filament diameter of 37 nm).[74,107,108] To account for this increase in thick filament diameter, Winegrad suggested that on phosphorylation of the MyBP-C motif, the postulated interaction between C1-C2 and C8-C9 would be disrupted because of additional negative charge. Thus, the cMyBP-C domains would shift, resulting in an interaction between domains C0 and C10 and expansion of the collar.

Squire et al[103] have recently proposed a model for MyBP-C binding to the thick filament (Figure 6) to include the potential interaction of MyBP-C with actin. In this model, the C-terminal domains of MyBP-C bind axially along the length of the thick filament, and the N-terminus extends perpendicularly toward the thin filament. Three MyBP-C molecules are arranged in this fashion around the same point in the myosin filament. This model takes into account the fact that MyBP-C domains C8-C10 are involved in titin binding[41] by suggesting that the two proteins can run parallel to each other. However, as discussed earlier, titin may well bind to only one or at most two of the three C-terminal domains of MyBP-C[41]; thus, a parallel arrangement is not necessarily required to account for the known interactions between MyBP-C, LMM, and titin. The Squire model also does not incorporate the C5:C8 interaction.

We have also proposed a model for cMyBP-C binding to the thick filament in a trimeric collar arrangement[98] based on our initial observation of an interaction between C5 and C8 (Figure 7). Supported by evidence of an additional interaction between domains C7 and C10,[98] we believe there are intermolecular interactions between staggered parallel cMyBP-C molecules encircling the myosin rod, where domains C5-C10 in each molecule are involved in forming the collar, whereas domains C0-C4 extend into the interfilament space and interact with (at least) myosin S2. Such a model is consistent with early structural characterizations of MyBP-C.[2,11] The minimum circumference of the collar would be the length of nine Ig domains, although it is likely to be longer because of the presence of interdomain linkers and the possibility that the alignment of the interacting domains is offset. Given that the length of the long arm (presumably C5-C10) of the V-shaped structures seen by Hartzell and Sale[11] is ≈26 nm and that this would constitute two thirds of the circumference of the collar, the collar dimensions would fit with the backbone diameter of 13 to 15 nm stated by Squire et al[39] to include both myosin and titin. The collar may also account for the ring of mass at every third crown of myosin heads seen by Eakins et al.[109] Finally, it has been found that the addition of exogenous C5 to skinned trabeculae decreases contractility.[110] Because C5 does not to bind to myosin[34,68] or to actin,[104] and because the effect of exogenous C5 was independent of the phosphorylation status of endogenous cMyBP-C (and so unlikely to act via the N-terminal regulatory region of cMyBP-C), this effect seems likely to be due to disruption of the endogenous C5:C8 interaction. This lends weight to the theory that the C5:C8 interaction has an important physiological role. However, although we believe this model to be the likely arrangement of cMyBP-C on the thick filament, further confirmatory evidence is still needed.

How Might Perturbation of the Functions Ascribed to cMyBP-C Cause HCM?

It is possible that missense and truncation mutations in cardiac MyBP-C cause HCM via different genetic mechanisms, but we believe it is more likely that both cause disease by acting in a dominant-negative/poison polypeptide fashion. As discussed, mice heterozygous for truncating mutations seem to indicate that mutant protein is expressed and incorporated. If even small amounts of C-terminally truncated cMyBP-C are incorporated in the sarcomere, it is easy to imagine that they could disrupt MyBP-C collar formation, resulting in the incorrect positioning of the MyBP-C motif relative to myosin S2. A mutation that prevented expression of domains beyond C7 would result in a protein that could not contribute to a MyBP-C collar at all, but the impact of this on its ability to bind S2, and the effect on contractility, is unknown.

There is presently no mouse model based on a cMyBP-C missense mutation with which to establish the effect on protein incorporation and subsequent pathophysiology. It is likely that these mutations cause disease by affecting the ability of the molecule to regulate contraction. Missense mutations in the C-terminal domains involved in the collar structure may affect the interactions between cMyBP-C molecules and disrupt the integrity of the collar, as has been suggested for the R654H and N755K mutations in C5. If one role of the collar is to correctly position the N-terminus for S2 binding, then with a disrupted collar and incorrect positioning of the MyBP-C motif, S2 binding may not occur, even in the absence of phosphorylation. Crossbridge cycling would presumably then occur without the brake usually applied by cMyBP-C.

Mutations in HCM disease genes have been shown to result in either hypercontractility or hypocontractility of the sarcomere.[111] Mutations at different locations in the β myosin heavy chain gene can result in opposing effects.[112] It is difficult to reconcile how these two mechanisms might lead to the same disease phenotype, but one hypothesis is that different classes of mutations all result in an increase in the cost of force production sufficient to cause energetic compromise.[113] Few empirical data exist to compare the physiological consequences of cMyBP-C mutations at the myofilament level. A transgenic mouse overexpressing cMyBP-C truncated in the C5 domain results in fibers with increased calcium sensitivity and decreased maximum force,[92] possibly because of an inability to anchor the S2 as efficiently as in wild-type mice fibers. The missense and truncation mutations in cMyBP-C might increase contractility by disrupting the interaction with myosin S2 and releasing the brake on crossbridge cycling. On the other hand, truncation mutations that result in cMyBP-C being unable to form the thick filament-tethering collar might decrease contractility by binding to S2 and inhibiting the actomyosin ATPase. Thus, either hypercontractility or hypocontractility could be plausible outcomes, but potentially both could result in energetic compromise.

Conclusion

There is increasing evidence that cMyBP-C plays an important dual role in the control of cardiac muscle contraction, both in the stabilization of the thick filament via the collar formation of the cMyBP-C C-terminal domains and in regulating the number of myosin heads available for involvement in the contractile cycle. The fact that mutations in the gene encoding this protein are a common cause of HCM has highlighted its importance in the cardiac sarcomere and has initiated informative structure:function analyses. As yet, neither the precise arrangement of MyBP-C in the sarcomere nor its role in thick filament stability and regulation of contractility has been completely elucidated. Emerging new data suggest that there are further complexities to be resolved.

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