Identification of Novel Interactions Between Domains of Myosin Binding Protein-C That Are Modulated by Hypertrophic Cardiomyopathy Missense Mutations

Johanna Moolman-Smook, Emily Flashman, Willem de Lange, Zhili Li, Valerie Corfield, Charles Redwood, Hugh Watkins

Abstract—Cardiac myosin binding protein-C (cMyBPC) is a modular protein consisting of 11 domains whose precise function and sarcomeric arrangement are incompletely understood. Identification of hypertrophic cardiomyopathy (HCM)–causing missense mutations in cMyBPC has highlighted the significance of certain domains. Of particular interest is domain C5, an immunoglobulin-like domain with a cardiac-specific insert, which is of unknown function yet is the site of two HCM-causing missense mutations. To identify interactors with this region, a human cardiac cDNA library was screened in a yeast two-hybrid (Y2H) assay using the C5 sequence as bait. Screening surprisingly revealed that domain C5 preferentially bound to clones encoding C-terminal fragments of cMyBPC; the interacting region was narrowed to domain C8 by deletion mapping. A surface plasmon resonance assay using purified recombinant cMyBPC domains was used to measure the affinity of C5 and C8 in vitro (Ka = 1×10^7 mol/L). This affinity was decreased about 2-fold by the HCM mutation R654H, and by at least 10-fold by the mutation N755K. Further Y2H assays also demonstrated specific binding between domains C7 and C10 of cMyBPC. Based on these novel interactions, and previous biochemical and structural data, we propose that cMyBPC molecules trimerize into a collar around the thick filament, with overlaps of domains C5-C7 of one cMyBPC with C8-C10 of another. We speculate that this interaction may be dynamically formed and released, thereby restricting or favoring cross-bridge formation, respectively. We suggest that the HCM mutations act by altering the cMyBPC collar, indicating its importance in thick filament structure and regulation. (Circ Res. 2002;91:704-711.)

Key Words: cardiac myosin binding protein-C ■ hypertrophic cardiomyopathy ■ missense mutations ■ ligand binding

Myosin binding protein-C (MyBPC) was first identified as a contaminant of muscle myosin preparations in the 1970s and has since been shown to be an essential component of vertebrate striated muscle. Immunolocalization studies have shown that MyBPC is bound to thick filaments in distinct stripes (between 7 and 11) in the C-zone of the A-bands in each half-sarcomere. Its precise quaternary structure on the myosin filament, however, remains controversial, as does its function, with both structural and regulatory roles proposed.

Fast and slow skeletal muscle isoforms of MyBPC, and a single cardiac isoform, are each encoded by a separate gene. The sequences of these genes reveal that MyBPCs consist of repeated domains with homology to immunoglobulin C2 (Ig-like) or fibronectin type 3 (FN3) domains (Figure 1). The first and second Ig-like domains (C1 and C2) are separated by a unique 103-residue stretch, termed the MyBPC motif, while linkers of varying sizes separate other domains (Figure 1). Cardiac MyBPC (cMyBPC) differs from the skeletal isoforms in that it contains an additional amino-terminal Ig-like domain (C0), as well as an insertion of LAGGGRIRS residues and three cardiac isoform-specific phosphorylation sites in the MyBPC motif, and a proline/charged residue-rich insertion into domain C5 (Figure 1).

Knowledge of the gene and protein structure facilitated investigation of the roles of MyBPC in the sarcomere and led to elucidation of the functions of specific domains. A structural role was first confirmed when the primary myosin and titin binding regions were localized to within C10 and C8-C10, respectively. It was also shown that, for MyBPC to be correctly incorporated into the thick filament, domain C7 is required in addition. A regulatory role for the cardiac isoform was indicated by a correlation between the rate of twitch relaxation and the catecholamine-sensitive phosphor-
ylation of cMyBPC.6,10 It has since been shown that the MyBPC motif of cMyBPC can bind to myosin-S211 and that this association is abolished by cAMP-dependent protein kinase phosphorylation of the MyBPC motif.12 This may provide a mechanism for regulating the attachment of myosin heads to actin and altering the contractility of muscle fibers. The degree of phosphorylation of cMyBPC also correlates with changes in thick filament backbone diameter and the degree of order of the myosin cross bridges, and so likely affects the probability of cross-bridge formation.13,14 It has also been proposed that domain C0 sterically hinders the myosin head; thus, mechanically constraining cross-bridge movement and providing cardiac muscle with yet another mechanism to regulate the development of force.15

Interest in the function, structure, and interplay of sarcomeric proteins has increased with the recognition that HCM is a disease of the sarcomere.16 This clinically and genetically heterogeneous disease is caused by more than 150 different mutations in 9 different sarcomeric protein genes.17 Mutational analysis has helped elucidate the pathophysiology of the disease, by directing in vitro and in vivo investigations of the effects of these mutations (see review18). Mutations in cMyBPC are responsible for a clinically recognizable subclass of HCM19,20 characterized by late onset hypertrophy that progresses throughout life.21 Most of the mutations in MYBPC3 are splice-site or deletion/insertion mutations that lead to truncation of the protein17 (see review 18) with loss of the C-terminal titin and/or myosin binding sites. It has been suggested that these may affect the incorporation of cMyBPC into the sarcomere, a notion that has been largely confirmed by a number of studies.22,23 However, at least 14 missense mutations in MYBPC3 have also been reported to cause HCM.21,24–27 Although some of these cause amino acid substitutions within the known myosin and titin binding domains, a number occur in domains of unknown function. These “experiments of nature,” the individually rare disease-causing mutations, direct attention to areas of the protein not previously known to be functionally important.

Two such missense mutations, R654H24 and N755K,26 have been described in domain C5 of cMyBPC, occurring on either side of the 28 amino acid cardiac-specific insertion. This suggests that this region has an important role in cMyBPC structure/function in the heart. We hypothesized that domain C5 of cMyBPC, being an Ig-like domain, might interact with one or more specific binding partners and so used the yeast two-hybrid (Y2H) system to search systematically for cardiac-expressed ligands. We identified, unexpectedly, an interaction between domains C5 and C8 of cMyBPC and subsequently used surface plasmon resonance to confirm the interaction and to demonstrate the impact of the known HCM-causing missense mutations in C5 on ligand binding. We have used our data along with previous biochemical and structural findings to propose a revised model for the arrangement of cMyBPC in the sarcomere based on cMyBPC multimerization.

Materials and Methods

Bacterial and Yeast Strains

Saccharomyces cerevisiae strains PJ69-2A and Y187 were used in the Y2H library screen, while S. cerevisiae strain AH109 was used in some Y2H direct protein-protein interaction assays. Escherichia coli KC8 was used to isolate prey plasmids from yeast (all strains from Clontech).

Constructs

Baits

The region of MYBPC3 encoding domain C5 was PCR-amplified from a MYBPC3 cDNA clone from an adult cardiac λZapII cDNA library (Stratagene No. 936208) and cloned in-frame with the GAL4-DNA binding domain (GAL4BD) in pAS2.1 (pAS2.1-C5). The region of MYBPC3 encoding domain C7 was PCR-amplified from the MYBPC3 cDNA clone and the product cloned in-frame with the GAL4BD in pGBK7 (pGBK-C7).

C5 bait constructs containing either the R654H24 (pAS2.1-C5H654) or the N755K26 (pAS2.1-C5K755) point mutations were
generated by PCR-based site-directed mutagenesis, as previously described,28 with fragments cloned in-frame with GAL4-BD in pAS2.1.

The integrity of the cloning sites, reading frame, and MYBPC3 sequence of all constructs were verified by bidirectional sequencing.

Preys

**Cardiac cDNA Library**
A pretransformed MATCHMAKER library (Clontech), consisting of *S. cerevisiae* strain Y187 transformed with a cardiac cDNA library constructed in pACT2, was used for Y2H library screening.

**cMyBPC**
Prey constructs encoding fusions of specific cMyBPC domains and GAL4-BD were generated by PCR-amplification of the MYBPC3 region(s), followed by in-frame cloning of these products in pACT2.

**Yeast Two-Hybrid Assay**

**Library Screening**
The MATCHMAKER two-hybrid system 2 (Clontech) was used according to the manufacturer’s protocols. Briefly, pAS2.1-C5 was transformed into *S. cerevisiae* strain P169-2A, and this bait line was mated with the *S. cerevisiae* Y187 pretransformed cardiac cDNA library line. Diploid clones were grown on medium lacking leucine (Leu), tryptophan (Trp), and histidine (His) (TDO plates) for 10 days, and then transferred to medium lacking adenine (Ade) as well as Leu/Trp/His (QDO plates) for a further 8 days. Colonies positive for the reporter genes ADE2 and HIS3 were tested for expression of the reporter gene LacZ by β-galactosidase filter assay.

Colonies expressing all reporter genes were further analyzed: duplicate interactor clones were identified by *HoeII* digestion profiles of nested PCR products from the prey plasmid inserts. Prey plasmids from representative clones were rescued from diploid yeast cells via *E. coli* KC8 cells, retransformed into *S. cerevisiae* Y187, and tested for activation of reporter genes autonomously or in the presence of heterologous baits. Putative true interactors were sequenced and the sequences analyzed with BLASTN (GenBank). The deduced amino acid sequence of open reading frames (ORFs) in-frame with the GAL4AD were analyzed with BLASTP.29

**Deletion Mapping of Interacting Domains**
Prey constructs of fusion genes encoding individual domains, or combinations of domains, of cMyBPC and GAL4-BD in pACT2 were individually transformed into *S. cerevisiae* Y187. These lines were mated with the bait line (pAS2.1-C5) transformed into *S. cerevisiae* AH109 and diploid cells selected on medium lacking Leu and Trp. These colonies were replicated to TDO plates (2 to 4 days); thereafter, interacting clones were selected on QDO plates (6 days).

**Expression and Purification of cMyBPC Domains in E. coli**
Expression constructs (either pMW17230 or pET28a; Novagen) were assembled encoding the wild-type domains C5, C5-C7, C4, and C8-C10 and mutant C5 and C5-7 domains containing the R654H and N755K amino acid substitutions. Recombinant proteins were overexpressed and isolated by standard chromatographic techniques at a final purity of >95%.

**BLAcore Experiments**
Proteins were diaлизed into myosin binding buffer, and binding studies were performed on a BLAcore X biosensor (Biacore AB). The C8-C10 fragment was covalently coupled to one flow cell of a B1 sensor chip using the BLAcore Amine Coupling Kit (BIAcore AB) at a buffer flow rate of 20 μL/min, 25°C, until the response units had increased by between 1000 and 2000. The control flow cell underwent the same treatment without addition of C8-C10. Proteins were injected over both flow cells for 150 seconds at the same flow rate. Equilibrium binding constants were calculated for C5 wild-type, R654H, and N755K (His-tagged) and C5-C7 wild type, R654H, and N755K (all His-tagged) using the BIAevaluation software (BIAcore AB). Repeat experiments with C5 domains using all His-tagged proteins from the pET-28a constructs demonstrated comparable binding patterns.

An expanded Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

**Results**

**Library Screening With C5 Bait**
Screening more than 7 x 10^6 cDNA clones with domain C5 of cMyBPC as bait, using a yeast mating-based Y2H analysis method, yielded 13 independent interactor clones prototrophic for Ade, as evidenced by robust light pink to white colonies on QDO medium and a positive β-galactosidase filter assay within 3 hours (Table). Eleven of these clones, 7 of which were represented by additional, apparently identical, cDNAs (Table), showed no activation of the three reporter genes autonomously or in the presence of heterologous baits. BLASTN comparisons of the nucleotide sequences derived from representative prey cDNAs of these 11 interactors revealed significant matches to genomic sequences in GenBank (Table). However, 10 were discarded as being implausible, because the predicted in-frame ORFs encoded either very short peptides with no significant database matches, or proteins that are physically separated from cMyBPC in vivo (Table).

The remaining clone, designated clone 77, was the most abundantly represented among the positive clones (1 of 4 apparently identical clones), and gave the strongest and fastest lacZ expression on β-galactosidase filter assay. Sequencing of clone 77 revealed that it encoded the C-terminal 608 amino acids of cMyBPC itself, from residue 667, which is located within the C5 domain, through to the stop codon.

**Deletion Mapping of C5 Ligand**
To define the domains involved in this interaction and to confirm specificity, deletion mapping of this region of cMyBPC was undertaken. Diploid yeast cells harboring both pAS2.1-C5 and pACT2-C5 were unable to activate any of the reporter genes, but were able to activate all three reporter genes when pAS2.1-C5 and pACT2-C6-C10 were present (Figure 2A). This ability was retained in the absence of C10, as seen with pACT2-C6-C9 (Figure 2A). Further deletion mapping of the C6-C10 region demonstrated that all deletion constructs that included domain C8 were able to activate the reporter genes in the presence of pAS2.1-C5, but could not do so autonomously (Figure 2A). In contrast, constructs lacking domain C8 failed to activate the three reporter genes, whether in the presence or absence of pAS2.1-C5 (Figure 2A). These results are summarized in Figure 2B.

**Direct Protein-Protein Interaction Assay of C7**
Using the results obtained from the C5-library screen, we proposed an arrangement of cMyBPC in the sarcomere based on an overlapping, parallel, multimerization bringing C5 of one molecule into contact with C8 of the next (see Discussion). In such an arrangement domain C7 of one cMyBPC molecule would be juxtaposed to domain C10 of another. To establish whether specific interaction also occurred between these two domains, an Y2H direct protein-protein interaction
calculation of the association equilibrium constant ($K_a$) for this interaction. For wild-type C5, the $K_a$ was 9.83 (n=3). There seems therefore to be a stronger interaction.

0.09

106 mol/L

102 mol/L

of the association ($k_a$) and dissociation ($k_d$) rate constants and dissociation phases of the binding curves allowed estimation of this interaction was such that the $K_a$ can only be approximated at 8.68×10^3 mol/L$^{-1}$ (n=3). The interaction was shown to be specific to the C5 domain, as C4 (the flanking Ig-like domain in CMyBPC) does not bind significantly to C8-C10 (Figure 4B). Both the C5 R654H and N755K mutants showed a significantly weaker binding to C8-C10 compared with wild type; the weakness of this interaction was such that the $K_a$ can only be approximated at 8.68×10^3 mol/L$^{-1}$. Interestingly, when C5-C7 (rather than C5 alone) was passed over C8-C10, the $K_a$ increased to 1.41±0.09×10^4 mol/L$^{-1}$ (n=3). Binding of C5-C7 R654H was not significantly weaker than wild type, with a $K_a$ of 1.36±0.07×10^4 mol/L$^{-1}$ (n=4); however, C5-C7 N755K bound with a $K_a$ of just 6.15±1.41×10^3 mol/L$^{-1}$ (n=3). There seems therefore to be a stronger interaction between this larger fragment and C8-C10, which is also affected by the HCM-causing mutations, though to a lesser extent than for C5 binding alone. This is consistent with the additional interaction between C7 and C10 demonstrated by the Y2H analysis.

**Discussion**

In the present study, we used Y2H analysis to identify a ligand for domain C5 of cMyBPC and found, unexpectedly, that the binding partner was cMyBPC itself; the binding sequence lay within the C-terminal domains (C5-C10). The region of interaction was subsequently narrowed down to domain C8 by deletion mapping of the original interactor clone. Evidence for the involvement of C5 in cMyBPC:cMyBPC interaction was compelling for a number of reasons. First, when domain C5 was used as bait to screen more than 7×10^6 cardiac cDNA clones, it preferentially bound to C-terminal cMyBPC clones containing the C8 domain. Moreover, of the 11 representative interactor clones identified in the library screen, only clone 77 contained an insert with an extended ORF in-frame with GAL4AD and encoded a protein whose in vivo localization was compatible with that of the bait. Reporter gene activation by clone 77 was specific to the pAS2.1-C5 bait and could not be elicited by heterologous bait constructs.

A surface plasmon resonance-based assay was then developed to demonstrate C5-C8 binding in vitro. This methodology has been frequently used to study the binding interactions of antibodies and cell surface receptors containing Ig domains. With the recombinant fragment C8-C10 immobilized on the chip surface, wild-type recombinant C5 bound with an estimated $K_a$ of $\approx$1.0×10^3 mol/L$^{-1}$. This is a comparatively weak interaction but is of a similar affinity to the association of other Ig-like domains measured by the same methodology, for example NCAM IgI and IgII. A binding constant of this magnitude is fully compatible with interaction of these domains in vivo, as in the thick filament the effective
concentration of cMyBPC will be high due to its attachment via its myosin and titin binding sites.

Previous investigations have suggested that both skeletal and cardiac isoforms of MyBPC multimerize. At physiological ionic strength, the sedimentation coefficient of skeletal MyBPC increased with rising concentration, consistent with dimerization of the protein and also with formation of higher multimers. Furthermore, cMyBPC eluted faster on gel filtration than would be expected on the basis of its chain molecular weight, indicating that the protein was asymmetric and/or rapidly and reversibly dimerised.

The dimensions of the v-shaped particles of purified cMyBPC (arm lengths 22.1 ± 4.5 nm), as determined by electron microscopy of rotary shadowed protein, suggest that the hinge of the v-shape most likely coincides with the region between domains C4 and C5; the presence of additional residues between these two domains also suggests that this is a flexible “linker.” Domains C5 and C8 are, therefore, expected to occur on the same arm, such that any cMyBPC:cMyBPC interaction appears more likely to be inter- than intramolecular. If the interaction is, indeed, intermolecular this could involve parallel or antiparallel orientation of the cMyBPC proteins. The orientation would determine which additional domains are in register, and which might, therefore, interact to stabilize the dimer structure. In an antiparallel arrangement, it is plausible that domains C5 and C8 of each cMyBPC would interact with domains C8 and C5, respectively, of the other. Such an orientation would also place domain C7 of each cMyBPC in register with domain C6 of the other. However, in a parallel arrangement, domains C7 of one cMyBPC would be in register with domain C10 of the other. To test these alternatives, domain C7 was used as bait in Y2H-based direct protein-protein interaction assay against other C-terminal cMyBPC domains. Only domain C10 demonstrated binding to C7, supporting a staggered parallel arrangement of cMyBPC during multimerization.

Early data suggested that the amount of MyBPC in the thick filament corresponds to 2 to 4 molecules per every third crown of myosin heads in the C-zone of the sarcomere. It would seem most likely that the number of cMyBPC molecules would be three, considering that myosin rods are most probably arranged in a three-stranded helix, that six, prob-

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<th>Construct</th>
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<tr>
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<tr>
<td>pACT2-C6-C9</td>
<td>C6, C7, C8, C9</td>
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Figure 2. A. Yeast two-hybrid reporter gene activation by different prey cMyBPC domains paired with bait domain C5. Growth of diploid yeast on QDO medium indicates interaction of C5 with a prey protein, leading to activation of the Ade2 and His3 reporter genes. Only prey constructs that included domain C8 of cMyBPC activated these reporter genes in the presence of domain C5. B. Summary of interactions of cMyBPC domain C5 with other carboxy-terminal domains of cMyBPC. Domains of cMyBPC encoded by the prey plasmids are schematically illustrated, whereas His⁺, Ade⁺, lacZ⁺ indicate the ability of these domains, in conjunction with domain C5, to activate all three reporter genes.
ably paired, titin strands lie along the surface of the myosin-backbone,\(^{33,34}\) and that the position of domain C10, which binds to both titin and myosin, would be dictated by the register of the first Ig-like domain of the titin superrepeats and the MyBPC binding site on the LMM component of myosin.\(^{7,8}\) Indeed, many different models of the arrangement of MyBPC in the thick filament have been based on three previous structural data. The point at which the proposed N-terminal arms veer away from the C-terminal collar coincides with the hinge of the v-shaped purified cMyBPC particles.\(^{32}\) The length predicted for these N-terminal arms (~26 nm) would be sufficient for the MyBPC motif to reach the S2 domain of myosin from the preceding crown. The length of each Ig-like domain and FN3 domain has been estimated at approximately 4 nm,\(^{33}\) and thus, the expected circumference of the proposed 9 domain collar is broadly compatible with the measured diameter of 13 to 15 nm for the intercross-bridge spaces of myosin filaments.\(^{33}\) In addition to the dimensions of the MyBPC domains, factors such as the structure of the linker amino acids between domains\(^{7}\) and the possibility that the alignment of the interacting domains is staggered may also influence the actual circumference of the proposed collar. Recent studies have suggested that when cMyBPC is phosphorylated the thick filament diameter may increase, altering the position and increasing the degree of order of the myosin cross bridges, thereby potentiating their binding to actin.\(^{13,14}\) These data imply that the cMyBPC collar is probably not static, but may have to widen on phosphorylation, and that the C5:C8 and the C7:C10 interactions might have to be abrogated and reformed in a manner compatible with the rate of phosphorylation of the MyBPC motif.

The surface plasmon resonance data suggest that the two HCM-causing missense mutations in domain C5 significantly weaken the C5:C8 interaction and hence in vivo may perturb the structure of the proposed MyBPC collar. If a function of the collar is to provide the correct positioning of the myosin S2-binding N-terminal region of MyBPC with respect to the myosin neck, then its disruption may reduce this interaction in the absence of MyBPC phosphorylation. Mutations that disrupt the collar may therefore have an activating effect by favoring cross bridge formation. In this way, these mutations may increase the rate of ATP consumption as has been seen for other classes of HCM mutations that appear to act by increasing the cost of force production.\(^{18,42}\) The degree of disruption of the C5:C8 interaction by the R654H and N755K missense mutations, as measured by surface plasmon resonance, appears to mirror the severity of clinical features associated with each. Although only described in a small
family, the R654H mutation was associated with low penetrance and only moderate hypertrophy even in adults, whereas the N755K mutation was associated with near complete penetrance, quite marked hypertrophy even in children, and sudden cardiac death in 1 of 8 affected patients.

The site of interaction of C8 on the C5 domain remains speculative. The R654H and N755K mutations may result in a reduced affinity for C8 either because they are directly involved in the interaction or because they induce a conformational change that is transmitted to the binding interface. Molecular modeling of this Ig-like domain suggests these two residues lie on the same surface of C5 and in close proximity to the 28 residue cardiac-specific insertion. It is tempting to speculate that this sequence may be important for modulating the C5:C8 interaction. Interestingly, a recent preliminary NMR study of C5 has suggested that this insertion forms a mobile region on the outside of the domain.

In conclusion, the observation of disease-causing mutations in a region of cMyBPC not previously associated with a particular function has highlighted novel structure/function aspects for this protein. The approach of integrating mutational data with functional and structural investigations of the relevant individual domains is likely to lead to important insight into both the quaternary structure of cMyBPC within the sarcomere and the manner in which it regulates muscle contractility in both health and disease.

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References


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