Binding of Myosin Binding Protein-C to Myosin Subfragment S2 Affects Contractility Independent of a Tether Mechanism

Samantha P. Harris, Elena Rostkova, Mathias Gautel, Richard L. Moss

Abstract—Mutations in the cardiac myosin binding protein-C gene (cMyBP-C) are among the most prevalent causes of inherited hypertrophic cardiomyopathy. Although most cMyBP-C mutations cause reading frameshifts that are predicted to encode truncated peptides, it is not known if or how expression of these peptides causes disease. One possibility is that because the N-terminus contains a unique binding site for the S2 subfragment of myosin, shortened cMyBP-C peptides could directly affect myosin contraction by binding to S2. To test this hypothesis, we compared the effects of a C1C2 protein containing the myosin S2 binding site on contractile properties in permeabilized myocytes from wild-type and cMyBP-C knockout mice. In wild-type myocytes, the C1C2 protein reversibly increased myofilament Ca\(^{2+}\) sensitivity of tension, but had no effect on resting tension. Identical results were observed in cMyBP-C knockout myocytes where C1C2 increased Ca\(^{2+}\) sensitivity of tension with the half-maximal response elicited at ~5 \(\mu\text{mol/L}\) C1C2. Maximum force was not affected by C1C2. However, phosphorylation of C1C2 by cAMP-dependent protein kinase reduced its ability to increase Ca\(^{2+}\) sensitivity. These results demonstrate that binding of the C1C2 peptide to S2 alone is sufficient to affect myosin contractile function and suggest that regulated binding of cMyBP-C to myosin S2 by phosphorylation directly influences myofilament Ca\(^{2+}\) sensitivity. (Circ Res. 2004;95:930-936.)

Key Words: Ca\(^{2+}\) sensitization • myocardial contractility • cardiac muscle • cardiac myocytes • cardiomyopathy • transgenic mice

Myosin binding protein-C (MyBP-C) is a thick filament accessory protein that binds tightly to myosin.\(^1\) The strong binding properties of MyBP-C are the result of at least two separate binding domains on MyBP-C that interact with distinct regions of myosin.\(^2\)–\(^4\) The first site, located near the C-terminus of MyBP-C, binds to the light meromyosin (LMM) fragment of the myosin rod, whereas the second site, located near the N-terminus of MyBP-C, binds the S2 subdomain of myosin (Figure 1). Although both sites are likely to contribute to the localization and function of MyBP-C within the sarcomere, the N-terminus site is encoded within a unique sequence referred to as the MyBP-C motif that is conserved across MyBP-C isoforms and that appears to be important in regulating MyBP-C function. This is especially evident for the cardiac isoform of MyBP-C (ie, cMyBP-C), which possesses phosphorylation sites within the MyBP-C motif that are not present in skeletal muscle isoforms.\(^5\) Importantly, phosphorylation of these residues by cAMP-dependent kinase (PKA) abolishes binding of the motif to myosin S2.\(^6\)–\(^7\) Because the residues are phosphorylated in vivo in response to a variety of inotropic stimuli,\(^8\)–\(^10\) regulated binding of cMyBP-C to myosin S2 may represent a significant means of modulating cardiac contractility.

Although the functional consequences of cMyBP-C phosphorylation are still not well understood, there is increasing evidence to suggest that MyBP-C normally limits the force and/or velocity of contraction. For instance, biochemical extraction of the majority of the cMyBP-C from rat myocytes led to a reversible increase in Ca\(^{2+}\) sensitivity of tension and accelerated unloaded shortening velocity at submaximal activation.\(^11\)–\(^12\) Consistent with these observations, rates of tension redevelopment, loaded shortening velocity, and power output were all increased in permeabilized myocytes from cMyBP-C knockout mice lacking cMyBP-C.\(^13\)–\(^14\) The apparent repressive effects of MyBP-C at the cellular level may also contribute to whole-heart hemodynamics in that the time course of ventricular stiffening or normalized elastance was significantly accelerated in mice lacking cMyBP-C.\(^15\)

As a potential mechanism to account for the effects of MyBP-C to limit myofilament contractile properties, Hofmann and colleagues\(^11\) proposed a model where MyBP-C acts as an internal load within the thick filament that opposes shortening. According to this model, MyBP-C could give rise to an internal load by tethering myosin S2 to the thick filament thereby limiting myosin head position and/or mobility. Calaghan and colleagues\(^16\) expanded on the idea of a MyBP-C tether to account for the effects of exogenous myosin S2 to enhance contractility in reversibly permeabilized myocytes from rat ventricles. The authors proposed that

Original received June 9, 2004; revision received September 27, 2004; accepted September 27, 2004.
From the Department of Bioengineering (S.P.H.), University of Washington, Seattle, Wash; Cardiovascular and Randall Divisions (E.R., M.G.), King’s College London, London, UK; and the Department of Physiology (R.L.M.), University of Wisconsin, Madison.
Correspondence to Samantha Harris, PhD, Department of Bioengineering, Box 357962, University of Washington, Seattle, WA 98195. E-mail spharris@u.washington.edu
© 2004 American Heart Association, Inc.

Circulation Research is available at http://www.circresaha.org
DOI: 10.1161/01.RES.0000147312.02673.56

930
the S2 and LMM myosin binding sites of cMyBP-C act together to anchor the myosin heads to the thick filament. Accordingly, interventions that disrupt binding at either of the two anchor sites, such as phosphorylation of the MyBP-C motif or competition with exogenously added binding domains, would be predicted to free the myosin heads and increase contractility.

However, Kunst and colleagues\textsuperscript{17} concluded that binding of cMyBP-C to myosin S2 directly affects contraction based on experiments showing that cMyBP-C peptides containing the S2 binding domain increased contractility in skinned skeletal muscle fibers. These authors proposed that these effects of MyBP-C binding to myosin S2 occurred independent of binding to LMM. However, because endogenous MyBP-C was still present in that study, the possibility that effects of the added peptides were due in part to competition with native MyBP-C for binding to S2 could not be eliminated.

The aim of the current experiments was to use cardiac myocytes from cMyBP-C knockout mice lacking endogenous cMyBP-C\textsuperscript{13} to provide an unambiguous test of the hypothesis that binding of cMyBP-C to myosin S2 directly affects myosin contractility independent of binding interactions with myosin LMM. Results demonstrate that binding of cMyBP-C to myosin S2 per se is sufficient to affect myosin contractility and provide support for the idea that at least some effects of cMyBP-C on contraction occur independent of a tether mechanism.

**Materials and Methods**

**Transgenic Mice**

Cardiac MyBP-C knockout mice\textsuperscript{13} were maintained on an SV/129 background. Wild-type SV/129 mice were obtained from Taconic Farms (Germantown, NY) and adult mice (8 to 40 weeks) of either sex were used for experimental measurements. Care and handling of all mice was performed according to institutional guidelines approved by the Association for Assessment and Accreditation of Laboratory Care International (AAALAC).

**Skinned Myocardial Preparations**

Multicellular myocyte preparations were obtained as described\textsuperscript{18} and were permeabilized (skinned) for 30 minutes at room temperature in a relaxing solution containing (in mmol/L) 100 KCl, 10 imidazole pH 7.0, 5 MgCl\textsubscript{2}, 2 EGTA, 4 ATP, 250 μg/mL saponin, and 1% Triton X-100. Myocytes were then washed repeatedly in fresh relaxing solution without saponin or Triton X-100 and stored on ice until used in experiments done the same day.

**Experimental Apparatus, Solutions, and Protocol**

The apparatus for mechanical force measurements was similar to that described previously\textsuperscript{19} and consisted of a temperature-controlled stainless steel plate containing a series of wells positioned between a length controller (model 308B, Aurora Scientific) and force transducer (model 403A, Cambridge Technology). Skinned myocyte preparations were attached to the force transducer and length controller by tying their ends to two steel troughs connected to the transducer and controller as described.\textsuperscript{19} The assembly was then transferred onto the stage of an inverted microscope fitted with a 40× objective and video camera for imaging and assessment of sarcomere length. Sarcomere length was adjusted to 2.3 μm and all measurements were performed at 15°C.
Compositions of activating and preactivating solutions were as described.18 Isoelectric focusing of 10-phosphorylated C1C2 was performed by Kendrick Laboratories. Before use in force experiments, the C1C2 peptide was concentrated as described6 using the catalytic subunit of PKA from bovine heart serum albumin as standard. Phosphorylation of C1C2 was performed in some cases by BioRad DC (Lowry) protein assay with bovine serum albumin as standard. Peptide expression

A peptide containing domains C1 through C2 of cardiac MyBP-C was expressed and purified as previously described.5,7 The amino acid sequence and purity of the C1C2 peptide are shown in Figure 1. Two-dimensional gel electrophoresis of phosphorylated and unphosphorylated C1C2 was performed by Kendrick Laboratories according to the method of O’Farrell.20 Isoelectric focusing of 10-μg samples was performed using a pH gradient from 3.5 to 10, and samples were separated in the second dimension on a 10% acrylamide slab gel. Gels were stained with Coomassie blue for visualization of proteins.

Results

Effects of C1C2 Peptide on Ca2+ Sensitivity of Tension

Figure 1 shows the amino acid sequence of the expressed C1C2 protein and purified C1C2 protein after exchange into experimental buffer solutions. Figure 2A shows the effects of the C1C2 peptide on the tension-pCa relationship in wild-type myocytes. Addition of 10 μmol/L C1C2 to experimental relaxing and preactivating solutions resulted in a pronounced leftward shift of the tension-pCa curve, indicating an increase in apparent Ca2+ sensitivity of tension in the presence of the C1C2 peptide. Removal of the C1C2 peptide from experimental solutions and brief (15 minutes) washout of the peptide from the myocytes resulted in a complete reversal of effects with the tension-pCa relationship returning toward control or to the right of control curves. The reason for the apparent overshift of the washout curve with respect to the control relationship is not immediately apparent.

To determine whether the ability of the C1C2 peptides to increase Ca2+ sensitivity was dependent on interactions with endogenous cMyBP-C, effects of the C1C2 peptide on tension-pCa relationships were measured in cMyBP-C knockout myocytes. Figure 2B shows a control tension-pCa relationship measured in the absence of C1C2 for knockout myocytes. Consistent with previous observations,13,14 the control relationship was somewhat right-shifted relative to that measured in wild-type myocytes (pCa50 of 5.71±0.02 versus 5.78±0.02), although the difference was not statistically significant. However, addition of the C1C2 peptide to experimental solutions induced a leftward shift in the tension-pCa relationship that was comparable or greater to the shift observed for wild-type myocytes and that was fully reversed on washout of C1C2. The finding that the C1C2 peptide increased Ca2+ sensitivity in cMyBP-C knockout myocytes indicates that effects of the C1C2 peptide do not depend on the presence of endogenous cMyBP-C. Furthermore, as shown in Figure 3, 30 μmol/L C1C2 peptide had no effect on maximum force measured in the presence of saturating [Ca2+] (pCa 4.5). Thus, the ability of the C1C2 peptide to induce an increase in apparent myofilament Ca2+ sensitivity at submaximal [Ca2+] was not related to a relative reduction in maximum force–generating capabilities of the myocytes.

Dose-Dependence of C1C2 Peptide Effects

To determine the concentration of C1C2 that was effective in increasing Ca2+ sensitivity, cumulative dose-response curves
were constructed for wild-type and cMyBP-C knockout myocytes. An experiment consisted of a reference activation in a pCa 4.5 solution, followed by 15 minutes in relaxing solution (pCa 9.0) containing 30 μmol/L C1C2. After 15 minutes, maximal tension was measured in pCa 4.5 solutions containing 30 μmol/L peptide (values shown as bars). In control experiments (−), 30 μmol/L C1C2 was omitted from the pCa 9.0 and 4.5 solutions. Values for force normalized to cross-sectional area (kN/m²) for the reference activation and activation in 30 μmol/L C1C2 were 43.48±9.6 and 41.29±9.9, respectively (mean±SE). Values for the reference activation and control activation were 18.27±4.7 and 17.62±4.8, respectively (mean±SE).

Effects of Phosphorylated C1C2

Because binding of C1C2 to myosin S2 was abolished by phosphorylation at residues unique to cMyBP-C,6,7 we next investigated whether phosphorylation of C1C2 affected its ability to increase Ca²⁺ sensitivity. As shown in Figure 5A, phosphorylation of C1C2 by cAMP-dependent protein kinase resulted in a dramatic reduction in the efficacy of C1C2 to increase submaximal tension, with a shift in the logEC₅₀ values from 0.74±0.10 to 1.25±0.12. As shown by two-dimensional gel electrophoresis of C1C2 peptides before and after phosphorylation by PKA (Figure 5B), the shift corresponded to a change in isoelectric point of the C1C2 peptide from basic to acidic values.

Discussion

We used a peptide encoding the C1 through C2 domains of cMyBP-C to investigate functional effects of cMyBP-C binding to the S2 subfragment of myosin. Results demonstrate that the C1C2 peptide increased Ca²⁺ sensitivity of tension in myocytes from both wild-type and cMyBP-C knockout mice and that phosphorylation of C1C2 reduced its efficacy. The results thus indicate that binding of the
N-terminus of cMyBP-C to myosin S2 is sufficient to affect myosin contractile function independent of the presence of endogenous cMyBP-C and provide support for the idea that regulated binding of MyBP-C to myosin S2 plays an important functional role in the modulation of myocardial contraction.

Binding of cMyBP-C to Myosin S2 Directly Affects Contraction

A C1C2 peptide containing the MyBP-C binding site to myosin S2 increased submaximal force with EC<sub>50</sub> values of \( \approx 5 \) \( \mu \text{mol/L} \) in both wild-type and cMyBP-C knockout myocytes. These values agree well with a \( K_d \) determined by isothermal calorimetry for one-to-one binding of C1C2 to recombinant myosin S2 in solution (\( \approx 5 \) \( \mu \text{mol/L} \)) \(^6\) and suggest that the observed effects of C1C2 are mediated by direct binding to myosin S2 at a single site. The finding that the EC<sub>50</sub> was identical in wild-type and cMyBP-C knockout mice further suggests that competitive interactions of the peptide with endogenous MyBP-C, if present, are minimal under the conditions of our experiments.

Although the lack of competition with endogenous cMyBP-C was initially surprising, the behavior is consistent with the prediction that most myosin S2 is unbound in wild-type myocytes because MyBP-C occurs at a low stoichiometry with respect to myosin, ie, 1 MyBP-C for every 8 to 9 myosin molecules. \(^4,22\) Therefore, even assuming that 100% of endogenous cMyBP-C is bound to S2 in wild-type myocytes, the majority of myosin S2 is presumably free and available for binding C1C2. The close agreement between EC<sub>50</sub> values from wild-type and cMyBP-C knockout myocytes is consistent with the idea that C1C2 binds to unoccupied S2 and implies that little (\( \approx 10\% \) or less based on a stoichiometry of 9:1), if any, of the ability of the C1C2 peptide to increase force in the present experiments is an indirect result of competition with endogenous cMyBP-C for S2 binding. In further support of this conclusion, localization studies using fluorescently labeled C1C2 protein showed that the peptide bound uniformly across myofibril thick filaments, but did not bind to the bare zones of A bands, which lack myosin heads. \(^7\)

However, the data do not eliminate the possibility that combined interactions of the multiple myosin binding sites of cMyBP-C may be important for contraction in vivo. For reasons related to stoichiometry cited earlier, such interactions might be difficult to detect in the current experiments because direct effects of C1C2 binding to all available S2 binding sites could potentially mask more subtle contributions involving only endogenous cMyBP-C binding sites. For instance, Calaghan and colleagues \(^16\) found that purified S2 added to reversibly permeabilized myocytes increased Ca\(^{2+}\) sensitivity, presumably by competing with endogenous S2 for binding to cMyBP-C. Because this result is qualitatively opposite (ie, reduced binding of cMyBP-C to S2 increased Ca\(^{2+}\) sensitivity) to the results obtained in the present study, it is possible that the combined effects of cMyBP-C binding to myosin at two or more sites are distinct from the effects of binding to S2 alone. It will therefore be of interest to determine the relative contributions of the different binding sites, \(^23-25\) alone and in combination, to contractile activity and to determine whether one or more of the cMyBP-C binding sites act to physically constrain myosin S2 heads, ie, as a myosin tether.

Apart from the possibility of combined interactions among multiple binding sites, the present results indicate that C1C2 binding to myosin S2 alone is sufficient to affect contraction in cardiac myocytes. Kunst and colleagues \(^17\) reached a similar conclusion based on observations that the C1C2 peptide increased Ca\(^{2+}\) sensitivity of tension in permeabilized skeletal muscle fibers. However, in contrast to the results reported for skeletal fibers, maximal Ca\(^{2+}\) activated force was not significantly reduced by C1C2 in cardiac myocytes. Reasons for the apparent discrepancy between effects of C1C2 peptides are unclear, but could be related to myosin isoform type or other fiber type differences. If so, they may reflect important regulatory differences between the two muscle types.

The finding that phosphorylation reduced the ability of C1C2 to augment Ca\(^{2+}\) sensitivity of tension suggests that
regulated binding of the MyBP-C motif to S2 contributes to contractility in vivo where several adrenergic and cholinergic agonists are known to affect cMyBP-C phosphorylation. For instance, cMyBP-C is phosphorylated by PKA in response to adrenergic stimuli. Because phosphorylation eliminates binding of C1C2 peptides to myosin S2 in solution, regulated binding of the cardiac MyBP-C motif may be important in the modulation of contractility by inotropic agonists. Notably, the effects of C1C2 phosphorylation on force described in this study are in the appropriate direction to contribute to reductions in Ca$^{2+}$ sensitivity observed in response to adrenergic stimuli. That is, by reducing binding to S2, phosphorylation of C1C2 could be expected to shift Ca$^{2+}$ sensitivity to the right. Although the rightward shift following adrenergic stimulation has been primarily attributed to phosphorylation of troponin I, the current results raise the possibility that cMyBP-C also contributes to this response.

**Role of Myosin S2 in the Regulation of Contraction**

The finding that C1C2 binding to myosin S2 affects contraction independent of MyBP-C binding to LMM suggests that the S2 subfragment directly influences force generation as opposed to simply providing a passive anchor point that limits myosin head extension from the thick filament. The S2 subfragment of myosin corresponds to a proteolytically sensitive region of the myosin rod that links the two catalytic S1 heads to the insoluble coiled-coil tail, or LMM domain, of myosin. The distal segment of S2, ie, the region adjacent to LMM, contains the flexible “hinge” domain of myosin, whereas the proximal end of S2 encompasses the region where the two myosin heavy chains dimerize to form the coiled-coil helix. The binding site for C1C2 was localized within the proximal 126 amino acids of S2, ie, the region adjacent to the S1/S2 junction. Because characteristics of the S1/S2 junction, such as its rigidity, mobility, and orientation could all potentially affect the mechanical efficiency of force generation or interactions between S1 heads, binding of cMyBP-C to S2 could provide a means for regulating these properties. Similarly, there is evidence that dimerization of the myosin heavy chains may be a dynamic process. Because C1C2 binds to dimeric S2, it is possible that cMyBP-C binding stabilizes the coiled-coil conformation of S2 and thereby influences force development. Additional studies are necessary to distinguish between these possibilities, our results are consistent with a regulatory role for the S2 subfragment in contraction.

**Implications for Mechanisms of Disease**

The functional significance of cMyBP-C binding to myosin S2 is highlighted by the prevalence of disease-causing mutations that occur within the cardiac MyBP-C (MYBP-C3) and β-myosin heavy chain (MYH7) genes and that potentially could affect MyBP-C binding to S2. Mutations in cMyBP-C and MYH7 together account for >80% of the identified causes of inherited hypertrophic cardiomyopathy, and MYH7, the majority of mutations in cMyBP-C are splice site donor/acceptor or base pair insertions/deletions that result in the introduction of premature stop codons. Assuming that these faulty messages are translated into protein, the majority of cMyBP-C mutations are predicted to encode truncated peptides that possess intact N-termini but that are shortened to varying degrees starting at their C-termini. Because most of the shortened peptides contain the S2 binding site, delocalized expression of the S2 binding site could affect myosin contraction and lead to long-term compensatory effects.

However, truncated peptides have not yet been demonstrated in human hypertrophic cardiomyopathies and it is likely that if expressed, they are present in small quantities because of RNA surveillance mechanisms. In this regard, the present findings that C1C2 was effective at concentrations in the low micromolar range and that C1C2 can apparently affect all myosin molecules (not just those with endogenous cMyBP-C already bound) suggests that even small quantities of delocalized peptides may be important in causing cardiac dysfunction and remodeling. This idea is consistent with observations that even low amounts of transgenic expression of a truncated cMyBP-C peptide led to structural and functional deficits. Therefore, it will be of interest to determine the long-term effects of cMyBP-C binding to S2 in vivo as well as effects on contraction of other potential binding sites within the N-terminus of cMyBP-C.

**Acknowledgments**

This work was supported by the National Institutes of Health Grant PO1-HL47053 (to R.L.M.), an American Heart Association Scientist Development Grant 0130557Z (to S.P.H.), and the Medical Research Council of Great Britain (to M.G.).

**References**


14. Korte FS, McDonald KS, Harris SP, Moss RL. Loaded shortening, power output, and rate of force redevelopment are increased with knockout of cardiac myosin binding protein-C. Circ Res 2003;93:752–758.


Binding of Myosin Binding Protein-C to Myosin Subfragment S2 Affects Contractility
Independent of a Tether Mechanism
Samantha P. Harris, Elena Rostkova, Mathias Gautel and Richard L. Moss

_Circ Res._ 2004;95:930-936; originally published online October 7, 2004;
doi: 10.1161/01.RES.0000147312.02673.56
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/95/9/930

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/